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TITLE: Novel Tissue Models of Junctional Epidermolysis Bullosa to Characterize Functional Mechanisms of Sulfur Mustard Injury to Human skin

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### INTRODUCTION

A major reason for our limited understanding of what triggers SM injury is that detailed mechanistic studies using human skin have not been possible for ethical reasons. Therefore, we have used an approach that has allowed us to identify sites and pathways of sulfur mustard (SM)-induced vesication using engineered human skin that mimics the clinical and histologic features of this tissue. Through research conducted this past year, our laboratory has extensively studied the pathophysiology of bioengineered, in vitro, 3-D human skin in response to SM by establishing dose/time responses of these human, skin-like tissues that lead to dermal-epidermal separation. We have developed and adapted novel tissue models that have found that structured basement membrane can alter the response to SM injury by making the tissue less susceptible to SM-mediated damage. We used immunohistochemical, morphologic and biochemical analyses to characterize the influence of these ECM and BM subtrates on the morphogenesis, survival, differentiation and growth of NHK. We have found that the presence of individual BM components, Type IV and tissues grown on intact complete BM (deepidermalized dermis) in organotypic cultures supports the survival of these skin-like tissues. there is a significant linkage between the pathologic alterations in Junctional Epidermolysis Bullosa (JEB) and SM injury, our understanding of he molecular defects in in BM causing JEB should shed light on the pathogenesis of SM-induced blistering. To further explore the role of basement membrane proteins in keratinocyte resistance to SM-induced damage, we directly studied the role of laminin 5 in this process. Primary keratinocytes harvested from patients with the blistering skin disease Junctional Epidermolysis Bullosa (JEB), that lack a functional gamma 2 chain of laminin 5 and are not able to adhere to basement membrane, were transduced with retroviral vectors designed to restore laminin 5mediated adhesion. We found that only JEB cells in which laminin 5 adhesive function was restored (F-GAL) were resistant to apoptosis when exposed to SM (150 ug/ml), thereby implicating laminin 5mediated attachment as being important in limiting SM damage. These studies provide important proof of concept that in vitro and in vivo tissue models mimic many of the tissue alterations previously found in animal models of SM injury. Our findings show that adhesion to basement membrane proteins enables subsets of keratinocytes to resist SM damage. Since we have previously found that only specific subsets of basal keratinocytes underwent cellular damage leading to apoptosis in our in vivo engineered tissue models, we have taken an important step towards defining the ECM or BM components that provide survival signals that can protect cells when challenged with SM. These human tissue models will be of great relevance in understanding functional mechanisms of SM injury and in testing new countermeasures to limit its morbidity and mortality.

### **EXPERIMENTAL RESULTS:**

PART I: ESTABLISHMENT OF CONDITIONS UNDER WHICH SULFUR MUSTARD CAN ALTER THE SURVIVAL AND VIABILITY OF NORMAL HUMAN KERATINOCYTES AND JUNCTIONAL EPIDERMOLYSIS BULLOSA CELLS GROWN ON A VARIETY OF BASEMENT MEMBRANE COMPONENTS IN 2-D MONOLAYER CULTURES

TASK 1: To determine the dose/time responses of normal keratinocytes to sulfur mustard exposure

1. <u>Determination of the effects of ethanol on human keratinocytes:</u> Prior to the application of sulfur mustard (SM) on cultured cells, we conducted a series of experiments using various concentrations of pure ethanol to determine the effects of this solvent on keratinocyte growth and survival. This was done

because SM was dissolved in pure ethanol and it was important to determine if this vehicle would induce alterations in cultured cells at the SM concentrations needed. Experiments were carried out in p60 plates and keratinocytes were grown on feeder layers of  $\gamma$ -irradiated 3T3 cells until colonies were 60% confluent. Cultures were then exposed to different doses of ethanol (0.4%, 0.8%, 1.6%) for 30 min and compared to untreated controls. Plates were then rinsed three times with fresh media and were grown for an additional week. Colonies were stained using crystal violet and the diameter of colonies exposed to ethanol doses were measured. Based on our experiments we concluded that ethanol did not greatly alter the growth of keratinocytes compared to the non-exposed controls as the size of individual colonies was not altered upon exposure to ethanol (Figure 1).

2. Staining for cellular apoptosis to determine the dose and time response of cultured keratinocytes to sulfur mustard: To establish the SM doses that could induce alterations in keratinocyte growth, a dose and time study was performed to measure the response of human keratinocytes to SM. Keratinocytes were seeded on sterile coverslips with 3T3 feeder layers (10,000 cells/p60) for six days in p60 plates until the colonies became 70% confluent. Cultures were then exposed to different doses of SM and compared to ethanol-exposed controls. We initially selected four different doses 37.5, 75, 150, 300µM of SM and compared these to 0.25, 0.5, 1, 2% ethanol upon exposure for 7 min. Cells on coverslips that were exposed to agents were then processed 24 hours later for immunofluorescent staining for the presence of apoptotic cells using a monoclonal antibody that detects the cleavage product of keratin 18 that is the end result of apoptotic pathways (M30 Cytodeath-stain, Roche, Inc.). Fig. 3 shows the appearance of apoptotic cells that demonstrated M30 staining in the cytoplasm (red stain) for different doses of SM when compared to corresponding ethanol controls. At low SM doses of (37.5µM, 75µM), fewer apoptotic cells were seen when compared to 150 and 300µM SM doses. All doses of ethanol showed fewer apoptotic cells than SM-exposed cells. Thus a dose-dependent increase in the number of apoptotic cells was seen after SM treatment. Based on these findings, we selected 150 µM as a standard SM dose that induced a maximum number of apoptotic cells.

An additional experiment was performed to study the effect of the length of exposure to SM on cell survival and death. We selected a dose of 150µM to study the effects of SM exposure for 1,3, 7, 14, and 28 min. Staining was performed for apoptotic keratinocytes grown on coverslips using the M30 stain the numbers of apoptotic cells on 3 coverslips were counted for each length of exposure. A sharp increase in the number of apoptotic cells was seen for 7 min exposures when compared to 1 and 3 min exposures. Longer exposures (14 min and 28 min) resulted in an 8–10 fold increase in numbers of apoptotic cells when compared to cultures exposed for 7 min (Fig. 5). In comparison, untreated controls showed a very small number of apoptotic cells while ethanol showed very small changes in the number of apoptotic cells. Fig. 6 demonstrates the appearance M30 positive cell after exposure to SM and to 1% ethanol for 7 min. These findings demonstrated that a SM dose of 150µM at an exposure time of 7 min was sufficient to induce significant apoptotic cell damage in 2-D cultures of keratinocytes. As a result, it was decided to use 7 min as the standard SM exposure time for all future studies.

3. MTT viability/survival assays to determine keratinocyte survival after SM exposure: We next performed the MTT assay to evaluate the viability of normal keratinocytes after exposure to SM. In this assay, the yellow tetrazolium salt MTT is reduced in metabolically active cells to form insoluble purple formazan crystals that are solubilized by the addition of a detergent. The purple color can then be quantified by spectrophotometric means and provides a direct measure of cell viability upon exposure to SM. Conversely, a reduction in spectrophotometric measurement reflects the loss of cell viability. We

performed MTT assays using normal human keratinocytes (NHK) at different cell densities to establish if varying cell density could alter cell viability in response to SM. NHK's were plated at densities of  $5X10^4$ ,  $2.5X10^4$ ,  $1X10^4$  and  $1X10^3$  and exposed to either  $150\mu$ M of SM or to 1% ethanol control. Fig. 7 demonstrates that cells seeded at low densities (1,000 and 10,000 cells) showed no difference in cell viability when SM exposures were compared to ethanol controls (Fig. 7). However, at high cell densities (25,000 and 50,000 cells), SM exposure significantly decreased cell viability when compared to ethanol-exposed controls. This demonstrated that the sensitivity of detection of MTT assay required a threshold number of cells greater than 25,000 to yield differences in cell survival between SM- and ethanol-exposed cells. These studies laid the groundwork for all experiments by establishing parameters required for length and concentration of SM exposure needed to alter cell viability and to induce apoptosis.

TASK 8: Dose-time response to establish the role of basement membrane components to SM in 2-D cultures

4. The role of basement membrane components on the survival and viability of keratinocytes exposed to sulfur mustard - We next performed experiments to establish the importance of (BM) or extracellular matrix components (EMC) on cell viability and cell survival when exposed to different doses of SM compared to ethanol controls. To establish if the basement membrane (BM) protein Type IV collagen could alter the sensitivity of normal keratinocytes to SM, we seeded different numbers of keratinocytes on Type IV Collagen-coated, 24-well plates and performed MTT viability assays (Fig.8). Results were similar to those seen when cells were plated on tissue culture plastic as SM-exposed cells showed lower viability when compared to those ethanol-exposed at higher cell density (50,000 and 25,000). Similar differences were seen when cells were exposed to SM and ethanol after seeding onto Type I Collagen coated plates (Fig. 9). These experiments demonstrated that an SM dose of 150µM for 7 min reduced cell survival compared to ethanol exposure. Fig.10 presents MTT viability assays when 50,000 cells were plated on different substrates including plastic, Type I Collagen, Type IV Collagen, Fibronectin, Laminin and Poly D-lysine in monolayer, 2-D cultures. 24 hours after seeding, cells were exposed to 150µM SM or 1% ethanol for 7min. All the plates showed a decrease in cell viability when treated with SM compared to ethanol controls. However, the greatest reduction in viability (50%) upon SM exposure was seen for cells grown on Type IV Collagen. Since cells grown on this substrate also demonstrated the highest cell viability when exposed to ethanol, it is possible that the elevated cell growth on Type IV Collagen made cells more vulnerable to SM damage. Other substrates (Type I Collagen, Fibronectin and Laminin) demonstrated decreased viability upon SM exposure, but to a lesser degree than Type IV Collagen. Poly-D-lysine and plastic-coated dishes demonstrated the smallest loss of viability upon SM exposure. This may be due to the poor attachment of keratinocytes to poly D-lysinecoated plates compared to other substrates.

MTT assays were carried out for varied doses of SM and ethanol on different substrates (Fig. 11). Four different doses of SM were tested (37.5, 75, 150, 300μM) and were compared to controls (0.25, 0.5, 1, 2% ethanol) when cells were seeded onto tissue culture plastic, Type I Collagen, Type IV Collagen, Fibronectin and Laminin. As described above, the greatest decrease in viability after SM exposure was seen for cells grown on Type IV Collagen. Interestingly, this was the only substrate that demonstrated loss of cell survival even at low SM doses (37.5μM and 75μM). Other substrates showed no loss of viability for these low SM doses but did show a moderate decrease at higher SM doses. Thus, while ECM components were protective at low SM doses, they did not provide a survival advantage at higher SM doses. Significantly, cells grown on the BM component Type IV Collagen demonstrated the

greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures and intact BM may be required to mediate this event.

### TASK 5: Dose-time response to establish the response of JEB cells to low dose SM exposure

### 5. MTT Assay to determine the response of Junctional Epidermolysis Bullosa (JEB) cells to SM: There is a significant linkage between the pathologic alterations seen in Junctional Epidermolysis Bullosa (JEB) and those seen in vesicant injury induced by SM. To determine if cells lacking the ability to synthesize a functional laminin 5 molecule would demonstrate an altered sensitivity to SM exposure, we utilized primary keratinocytes that were derived from patients with JEB that were deficient in laminin 5 function. JEB cell lines were initially harvested from the skin of a patient with JEB (552) by the laboratory of Dr. Guerrino Meneguzzi (INSERM, Nice, France). Cells were infected with retroviral vectors that were previously shown in our lab (Progress Report 1) to modify laminin 5 function and restore cell-substrate adhesion in cells that were adhesion-deficient due to the absence of Gamma 2 chain (Phoenix producer cells courtesy of Nolan lab). The following vectors were used to modify laminin 5 function in 552 cells:

**Delta BC:** Cleaved variant of Gamma 2 chain of laminin 5 with a shortened 80 kd Gamma 2 chain. This generates cells that do not adhere well to connective tissue substrates.

**FGAL:** Non-cleaved variant of Gamma 2 chain. The chain remains intact and is not cleaved at it's BMP-1 site to generate a 155kd chain that restores adhesion.

**Pfu:** A Gamma 2 mutant that encodes cDNA for the constitutively cleaved form of this chain that has been truncated at the proteolytic cleavage site to generate a 105kd chain. These cells do not adhere well to connective tissue substrates.

Wild type (Gamma 2 WT): This has a full-length Gamma 2 chain and cells infected with this variant restore their laminin 5 function.

**Delta C115:** This is an empty vector that does not correct laminin 5 due to the absence of Gamma 2 chain and so that cells retain the properties of the mutant cells.

We used the MTT cell proliferation assay to study the effects of SM on JEB cells that were seeded on different BM and ECM components such as Type I Collagen, Type IV Collagen, Fibronectin and Laminin, as well as control plastic plates. Cells were seeded into 24-well plates on these substrates and exposed to 150µM of SM or 1% ethanol on the following day for an exposure time of 7min, rinsed three times with fresh media and incubated for an additional 24 h. Fig. 12 represents results of MTT assays for these JEB cells grown on different ECM or BM substrates. In general, there was an increase in cell viability for the Gamma 2 WT, Delta C115 and FGAL cells, demonstrating that these cells survived the exposure on different substrates to a greater degree when compared to Delta BC and Pfu. However, the degree to which cell viability was altered was dependent on the substrate on which the JEB cells were plated. For example, cells that had restored their laminin 5-mediated adhesive function (FGAL and Gamma 2 WT) showed a minimal loss of viability on Type I and Type IV Collagen and tissue culture plates when compared to JEB cells restored with a gamma 2 chain that did not support adhesion (Delta BC). However, SM-induced loss of cell viability was not as great with Delta BC-restored cells on Fibronectin as had been seen on other substrates. These findings demonstrated that restoration of adhesive function mediated by laminin 5 was able to provide decreased cell vulnerability and increased cell survival upon SM exposure.

In light of these findings, we next performed MTT assays for JEB cells on these substrates at an elevated dose (300 $\mu$ M) of SM and compared this to control exposures of 2% ethanol (Fig. 13). As a control, we compared the SM response of JEB cells to that of normal keratinocytes, as well. JEB cells whose laminin 5-mediated adhesion was restored (FGAL and Gamma 2 WT) showed a similar susceptibility to this dose of SM, as did cells whose adhesion was not restored (Delta BC). Interestingly, all cells grown on Type IV collagen showed a 2 to 3 fold decrease in cell viability even in the presence of 2% ethanol, suggesting that this substrate rendered cells more susceptible to cell death. Thus, it appears that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150 $\mu$ M) in 2-D culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300 $\mu$ M).

To further confirm these observations regarding threshold doses of SM, JEB cells were tested on the three substrates that showed the maximum difference in cell viability when treated with SM and ethanol. To accomplish this, JEB cells were exposed to doses of SM (75, 150, 300 μM) and corresponding doses of ethanol controls (0.5, 1, 2 %) for 7 min. Fig. 14 presents results of the MTT assay for the 5 different JEB cell types and human keratinocytes seeded on three different substrates (Type I Collagen, Type IV Collagen, Fibronectin) that were exposed to 75µM of SM or 0.5% ethanol. At lowest SM doses (75µM, 150µM), FGAL cells showed sensitivity to SM damage on Type I Collagen and Fibronectin, but not on Type IV Collagen (Fig. 14). This supported the observation that restoration of laminin 5-mediated adhesion and resistance to M damage were optimal on proteins present in the BM such as Type IV Collagen at low SM doses. This may occur as Type IV Collagen is known to interact with laminin 5 and may provide apoptosis resistance as due to this adhesive association. This also suggested that restoration of laminin 5 function will not reduce the sensitivity to SM damage when cells are grown on a substrate that is not found in BM, such as Type I Collagen. This apoptosis resistance seen for FGAL cells was lost at higher SM doses (300µM, Fig. 16) and was similar to that seen for cells that did not undergo restoration of laminin 5 function (Delta BC) at low SM doses on Type IV Collagen. However, the loss of viability for Delta BC cells was greater than that seen for FGAL cells on Type IV Collage substrates, even at the elevated SM dose (300µM).

A final experiment in monolayer, 2-D culture was performed to compare SM damage between Delta BC and FGAL cells on two substrates (Type I Collagen and Type IV Collagen) that showed the maximum difference in cell viability after SM and ethanol exposure (Fig. 17-20). MTT assays were carried out using 8 different SM doses (75, 150,300, 450, 600, 750, 900, 1200μM) and compared to their corresponding ethanol controls (0.5, 1,2, 3, 4, 6, 8 %). Both FGAL and Delta BC cells showed a threshold, SM dose below which no decrease in cell viability was seen. Surprisingly this threshold was highest (450μM) for Delta BC cells grown on Type IV Collagen plates. In contrast, FGAL cells grown on both Type IV collagen and Type I collagen, as well as Delta BC cells grown on Type I collage showed a small SM-induced decrease in cell viability below 150μM SM. However, as SM dose was increased to 1200μM a gradual dose-dependent decrease in cell viability was seen for both cell types on both Type I and Type IV Collagen substrates.

PART II: EXPOSURE OF THREE-DIMENSIONAL, HUMAN ORGANOTYPIC CULTURES HARBORING NORMAL HUMAN KERATINOCYTES AND JEB KERATINOCYTES TO DETERMINE THE ROLE OF BASEMENT MEMBRANE ON THE INDUCTION OF SULFUR MUSTARD INJURY

We next generated 3-D, organotypic cultures to identify pathways of SM induced vesication by using engineered human skin that mimics the clinical and histological features of this tissue. Organotypic cultures grown in the absence of pre-existing BM components ("Raft" cultures) were prepared according to our lab protocol. To accomplish this, early passage human dermal fibroblasts were added to neutralized Type I Collagen to a final concentration of 2.5X10<sup>4</sup> cells per ml. This mixture (3ml) was added to each 35mm well insert of a six-well plate and incubated for 4-6 days in media containing Dulbecco's Modified Eagle's Medium and 10% fetal calf serum, until the collagen matrix showed no further shrinkage. At this time, a total of 5X10<sup>5</sup> normal human epidermal keratinocytes were seeded directly on the contracted collagen gel. Alternatively, to generate cultures in the presence of BM, cells were seeded onto a de-epidermalized human dermis (AlloDerm) that was layered onto the contracted Type I Collagen gel. Organotypic cultures were maintained submerged in low calcium, epidermal growth media for 2 days, submerged for 2 days in normal calcium epidermal growth media and raised, to the air-liquid interface by feeding tissues from below with cornification media for an additional 2 days. At this point, cultures were exposed to different doses of SM or ethanol that were added to fresh media on day 7 of culture. Tissues were exposed for 7 min based on our previous results with 2-D monolayer cultures (see above). In addition to ethanol treated cultures, untreated cultures were used as controls by not adding ethanol to the media. After exposure, tissues were rinsed three times with fresh media and incubated for an additional 24 h. The following day, tissues were pulsed with 10µm bromodeoxyuridine (BrdU) 6h prior to terminating experiments to allow assay of proliferation in exposed tissues. Tissues were then bisected and one-half was snap-frozen in liquid nitrogen, while the other half of the tissue was formalin-fixed, paraffin-embedded and Hematoxylin and Eosin sections were prepared.

1. Establishing SM doses that induce tissue damage in 3-D organotypic cultures- morphologic and apoptotic alterations - To gain an understanding of SM doses needed to induce tissue damage in 3-D tissues, cultures were first treated with 75 and 150μm SM and compared to untreated cultures and those exposed to 1% ethanol. Figures 21 and 22 demonstrate the appearance of collagen Raft cultures grown in the absence of BM that were either unexposed (A), exposed to 75μM (B) or 150μM (C) SM or exposed to 1% ethanol (D). Untreated cultures generated a well-stratified epithelium that adhered to the underlying connective tissue. Tissues treated with 75μM SM demonstrated an intact epithelium that was well-attached to the underlying connective tissue (B). However, these tissues demonstrated a significant degree of altered tissue organization (B) that was similar to that seen for ethanol-exposed cultures (D). In contrast, cultures exposed to 150μM SM demonstrated complete dermal-epidermal separation (C) and an overall thinning of the epithelium. These preliminary findings showed that a 150μm dose of SM for 7 min could mimic the vesicating damaged induced by SM *in vivo*.

To assess the degree of apoptotic cell death in exposed tissues, M30 staining for Rafts exposed to different doses of SM and ethanol was performed from frozen sections. A four-fold increase in apoptotic cells was observed at a dose of 150μM of SM when compared to controls and twice as many apoptotic cells were seen with cultures treated with 75μM of SM (Fig. 23). We next determined if higher SM doses could increase SM-mediated tissue damage in Raft cultures by performing experiments using 150μM and 300μM of SM doses in comparison to 1 and 2% ethanol controls (Fig 24, 25). Low power magnification of H&E stained sections after treatment with 300μM SM showed a significant degree of tissue damage including complete separation of tissue from the BM zone as well as necrosis of keratinocytes and fibroblasts. Less damage was seen in tissues exposed to 150μM SM and there was no separation at the BM interface (Fig.25) To confirm these findings, frozen sections were stained using

the M30 antibody. Numbers of apoptotic cells were greatest in tissues exposed to 300µM SM while ethanol-treated tissues showed a very minimal number of apoptotic cells. These findings established that doses similar to those found to induce SM damage in 2-D cultures of kerationcytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our in vivo studies (REPORT Year 2).

### TASK 8: Dose-time response to establish the role of basement membrane components to SM in 3-D, organotypic cultures

2. Establishing the role of basement membrane in human skin response to sulfur mustard - In light of these findings, we next studied the effects of SM on organotypic cultures grown in the presence and absence of pre-existing BM components. This would allow comparison to MTT assays carried out on different BM components in 2-D cultures described above. Keratinocytes were seeded on AlloDerm, the de-epidermalized, acellular cadaver dermis derived from human skin that forms intact BM at its dermalepidermal interface. In this way, it would be possible to determine if BM can protect skin-like tissues from SM-induced damage when compared to tissues grown without BM components. To accomplish this, tissues were grown on AlloDerm, collagen Rafts or on polycarbonate membranes coated with either Type I Collagen, Type IV Collage, Fibronectin or control plastic. After 7 days in culture tissues were exposed to SM (150µM) and compared to tissues exposed to 1% ethanol. Fig. 27 represents the low power view of H&E stained tissue sections after SM exposure and Fig. 28 represents the higher magnification view. SM at a dose of 150µM induced separation at the BM zone when both collagen Raft (Fig. 27 B, Fig. 28 C) and plastic, non-coated inserts (Fig. 27 D, Fig. 28 B) were compared to the ethanol control (Fig. 27 D, E, Fig. 28 B, D). In contrast, tissues grown on AlloDerm showed that the BM interface was intact (Fig. 27 C, Fig. 28 E) as seen in ethanol-exposed controls (Fig. 27 F, Fib. 28F). Similarly, SM induced separation at the BM zone for tissues grown on different substrates (Type I Collagen, Type IV Collagen, Fibronectin) when compared to ethanol controls (Fig. 29 and Fig. 30).

To confirm these findings a dose of 150µM SM was used and compared to 1% ethanol and untreated controls to compare the effect of SM on organotypic cultures on which keratinocytes were grown on Rafts and AlloDerm (Fig. 31 and 32). Tissues treated with 150µM SM demonstrated intact tissues (Fig. 31 B) with minimal numbers of damaged, eosinophilic cells in the supra-basal layer (Fig. 32 B arrows) while untreated tissues showed a well-stratified epithelium that was similar to ethanol controls. In contrast, SM-treated Rafts showed a significantly higher number of damaged keratinocytes that displayed nuclear condensation and eosinophilic cytoplasm proving that SM could induce more severe damage to cells grown on Rafts than those grown on AlloDerm. To confirm these findings, we performed immunofluorescent staining using the M30 antibody to assess numbers of apoptotic cells in exposed tissues (Fig. 33 & 34). Rafts treated with SM showed a 10-fold increase in number of apoptotic cells when compared to AlloDerm (Fig. 34), which displayed number of apoptotic cells that were similar to non-treated and ethanol controls. To determine if the induction of apoptosis demonstrated a dosedependency, we performed experiments on AlloDerm using two different doses of SM along and compared them to controls. AlloDerms exposed to doses of 75µM and 150µM of SM were similar to untreated and ethanol controls in both tissue morphology (Fig. 35, 36) and upon M30 staining and numbers of apoptotic cells after M30 staining when compared to controls (Fig. 37).

In light of these findings, we next determined the minimal SM dose required for the induction of tissue damage in AlloDerm cultures. We selected 5 different SM doses (75, 150, 300, 600, 1200 μM) and compared these to ethanol controls (0.5, 1, 2, 4, 8 %) (Fig. 38 and 39). We found that the epithelium remained intact at SM doses of 75 and 150μM as induction of SM-mediated damage started at a dose of 300μM. At elevated doses of 600 and 1200μM SM tissue alterations were more prominent and were characterized by separation of the BM zone. Ethanol treated cultures showed little tissue damage even at elevated levels of ethanol exposure. M30 staining of these cultures showed a gradual increase in the number of apoptotic cells with increasing doses of SM from 75 to 300μM SM (Fig. 41). An SM dose of 75μM showed 3.8% of apoptotic cells compared to 57.2% observed for 1200μM while the maximum percentage of apoptotic cells observed for the highest dose of ethanol (8%) was only 3.6%. This gradual increase in numbers of apoptotic cells showed that AlloDerm tissues were susceptible to SM, but only above a threshold level of SM (600μM). These findings showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 uM.

- TASK 5: Dose-time response to establish the response of JEB tissue constructs which were reverted to a normal phenotype by retroviral gene transfer to SM
- TASK 6: Assay the response of JEB and normal organotypic cultures to high, vesicating doses of SM TASK 7: Assay the response of JEB keratinocytes expressing mutated forms of the gamma-chain of laminin 5
- 3. Establishing the role of laminin 5-mediated adhesion in the response of 3-D tissues to sulfur mustard the response of JEB mutants to sulfur mustard in 3-D tissues Since it appeared that BM could protect tissues exposed to SM, we performed a final experiment to determine if JEB cells grown on AlloDerm demonstrated a differential susceptibility to 150µM SM (Fig. 42 and 43). All JEB cells showed a degree of epithelial separation at the BM zone when compared to ethanol-exposed control cultures. Normal cells grown on AlloDerm showed protection from SM damage but AlloDerm did not protect JEB cells, (FGAL, Delta BC, Pfu, Delta C115 and Gamma2wt) against the SM induced damage. This was confirmed by M30 staining of these tissues where we observed the induction of apoptosis due to SM compared to ethanol controls (Fig. 45). Paradoxically, FGAL and Gamma 2 WT cells showed the greater number of apoptotic cells among all different JEB cell types. This suggests that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to 150µM SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM.

### **KEY RESEARCH ACCOMPLISHMENTS:**

- 1 We established dose/time responses of normal keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150uM) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
- 2 We established dose/time responses of JEB keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150uM) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
- 3 We have that established cells grown on the BM component Type IV Collagen demonstrated the greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that

individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures.

- 4 We have found that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150  $\mu$ M in 2-D) culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300 $\mu$ M).
- 5 We determined that doses similar to those found to induce SM damage in 2-D cultures of kerationcytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our in vivo studies (REPORT Year 2).
- 6 We have found that showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 uM.
- 7 We found that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to  $150\mu M$  SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM.

### **REPORTABLE OUTCOMES**

### **PAPERS**

- 1. Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J.A. Analysis of Microenvironmental Factors Contributing to Basement Membrane Assembly and Normalized Epidermal Phenotype, J. INVEST. DERMATOL. 120:923-931, 2003.
- 2. Greenberg, S., Prabhu, P., Garfield, J., Hamilton, T., Petrali, J., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004).

### ABSTRACTS PRESENTED

- 1. Prabhu, P., Greenberg, S., Lin, N., Garfield, J., Hamilton, T., Petrali, J., and Garlick, J.A. Characterization of the Initial Response of Engineered Human Skin to Sulfur Mustard (Society for Investigative Dermatology, 2004)
- 2. Kamath, P., Greenberg, S., Petrali, J., Hamilton, T., Garfield, J., Pommeret, O., Meneguzzi, G., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004)

### **CONCLUSIONS**

A major goal of our research studies was to determine the initiating site of SM induced damage that leads to vesicating injury in human skin. YEAR 1 of our research allowed us to generate optimized *in vitro* and *in vivo* human tissue models harboring basement membrane that have made many of the discoveries in this report possible. YEAR 2 of this research focused on characterization of the *in vivo* response of bioengineered human skin to prevesicating and vesicating doses of SM.

During this year of our research, we received approval for the use of an in-house facility for SM exposures. This laboratory was constructed during YEARS 1 and 2 of our research and final approval for its use was obtained one year ago. This facility greatly facilitated the progress of our work as it allowed all studies with SM to be performed in our laboratory. YEAR 3 of our research has allowed us to establish that intact basement membrane significantly reduces the vulnerability of 3D, human skin-like tissues to vesicating injury. The finding of an increased susceptibility of human skin-like tissues without structured basement membrane to SM-induced vesication indicates that this structure is a critical site for the initiation of SM injury in human skin. Studies in 2D cultures demonstrated that the presence of proteins found in the cutaneous basement membrane zone were not able to reduce the sensitivity to SM damage. In fact, quite the opposite was true, as we determined that cell viability (MTT assay) was lowered and apoptosis was increased (M30 assay) when cells were grown in 2D culture on the basement membrane component Type IV collagen and exposed to 150µM of SM. Since it is known that human epidermal keratinocytes have elevated growth on Type IV Collagen, it is possible that this increased proliferation was associated with the elevation of cell damage. Similarly, even in 3D cultures, the presence of basement membrane proteins that were not organized into structured basement membrane were not sufficient to protect keratinocytes from SM damage. We concluded that it was critical to have well-structured basement membrane in 3D (AlloDerm) cultures in order to prevent SM damage leading to vesication. To further establish the role of the basement membrane as a mediator of SM-induced vulnerability, we focused on the role of laminin 5. By using JEB cells restored with a variety of  $\gamma^2$  chain retroviral constructs, we concluded that restoration of adhesive function mediated by laminin 5 could decrease susceptibility to SM injury. These findings directly implicate laminin 5 and its role in structured basement membrane that mediate adhesive interaction that can prevent SMinduced vesication and can directly modulate SM injury in human skin.

# Chart 1:Colony forming efficiency test for diff. Doses of Ethanol.

1.2	Main   District   Di	Area         Diameter (mean)         Obj.#         Area           303         19.01095         1         723           297         18.57384         3         274           297         18.57384         4         497           719         29.17088         5         27.1           966         22.08782         7         20           968         22.08782         7         20           100         10.25018         8         1484           218         15.39338         10         784           218         15.39338         10         784           248         17.03653         12         753           248         17.03653         12         755           248         17.03653         14         302           248         17.03653         14         302           241         17.42212         17         302           252         17.48212         20         1359           240         20.34564         19         302           252         17.48212         20         1359           260         24.01897         26         1278	* 습 O	Area 368 1452 1369 1369 1369 1369 1369 1369 1369 1369		Area 693 2 695 695 695 695 695 695 695 695 695 695	Diameter (mean) 28.66456 26.8391 27.27014	0 to 4	333 457 572		- 46.	Δ '' '
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	22                 115                 115                 115                 115                 115                115                115                 115                 115                 115                 115                 115                 115                 115                 115                 115                 115                115                 115	17.48212         20         1359           31.67792         21         731           18.8583         23         314           21.10647         24         567           24.01897         25         127           24.03547         28         27         230           15.03547         28         847         143           14.35246         29         266         22           26.66582         32         1730         26           24.06542         33         813         193           19.79765         34         982         246           14.63457         40         908         246           14.63457         41         1714         20.51874         41         1714           20.51874         40.55771         45         343         315         365           20.51874         47         172         365         376         376         365         376         376         365         376         365         376         376         365         376         376         376         376         376         376         376         376         376         376         376		254 414 800 838 1027 1027	18402		23.38809	53		23	52	
7.0         1.0         7.0 <td></td> <td>31.67721       31.67721       31.67721       48.8583       22.10617       23.401897       23.62228       23.62228       25.03517       14.35246       25.65892       26.66892       27.06542       30.1730       15.55703       34.982       34.6547       40.997       31.0858       41.24627       42.3767       42.3777       42.5771       43.917       44       47.755       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756</td> <td></td> <td>838 838 1027 1027</td> <td>90816</td> <td>•</td> <td>36.99848</td> <td>8</td> <td></td> <td>go.</td> <td>88</td> <td></td>		31.67721       31.67721       31.67721       48.8583       22.10617       23.401897       23.62228       23.62228       25.03517       14.35246       25.65892       26.66892       27.06542       30.1730       15.55703       34.982       34.6547       40.997       31.0858       41.24627       42.3767       42.3777       42.5771       43.917       44       47.755       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756       47.756		838 838 1027 1027	90816	•	36.99848	8		go.	88	
		24.01897     21.07792       24.01897     24.01897       24.01897     24.01897       24.01897     24.01897       24.01897     25       15.0254     29       26.66582     26       26.66582     30       26.66582     32       26.66582     32       26.66582     32       26.66582     32       27.05542     34       31.07055     34       31.0858     41       26.66577     44       27.51874     44       47.75692     47       77.70592     736		838 838 1027 735	0.000		34.32692	31		<u>o</u>	27	
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100         100 <td>  13.   1.   1.   1.   1.   1.   1.   1.</td> <td>15.03517     28     847       14.35246     29     256       26.66592     32     196       24.06542     33     193       19.79765     34     982       12.24627     36     246       14.63457     40     908       31.08588     41     1714       23.7671     42     915       20.51874     44     1123       40.55771     45     343       17.70592     47     736</td> <td></td> <td>735</td> <td>.47375</td> <td></td> <td>18.10159</td> <td>66</td> <td></td> <td></td> <td>78</td> <td></td>	13.   1.   1.   1.   1.   1.   1.   1.	15.03517     28     847       14.35246     29     256       26.66592     32     196       24.06542     33     193       19.79765     34     982       12.24627     36     246       14.63457     40     908       31.08588     41     1714       23.7671     42     915       20.51874     44     1123       40.55771     45     343       17.70592     47     736		735	.47375		18.10159	66			78	
131 1437244         29 25 17 58 44 1 20 4 1 20	131   1,32426   2.9	14.35246     29     256       21.48697     30     1730       26.6582     32     196       24.0542     33     813       19.79765     34     982       15.5703     36     826       12.24627     36     246       14.63457     40     908       33.08588     41     1714       20.51874     42     915       40.55771     45     343       17.70592     47     736		7537	.40827		22 32742	9		: <b>s</b> c	. 92	
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469         26,66650         32         166,16257         36         461,167007         46         21,51600         36         461,167007         46         21,51600         36         461,167007         46         21,51600         36         462,16700         36         462,16700         36         462,16700         36         462,16700         36         462,16700         36         462,16700         36         462,16700         36         462,16700         46         21,51600<	66 50 50 50 50 50 50 50 50 50 50 50 50 50	26.66582     32     196       24.06542     33     813       19.79765     34     813       12.24627     36     826       14.63457     40     908       31.08588     41     1714       23.7671     42     905       20.51874     44     1123       40.55771     45     343       17.70592     47     736		1128	.05792		23.76301	: 4		! =	32	
666         200642         33         813         31,70016         36         21,7033         35         613         31,70016         36         21,7033         36         613         21,7036         46         21,7036         47	465         CALONSEC         35         615         317001         36         217001         36         217000         36         317000         36         317000         36         317000         36         317000         36         317000         317000         36         3170000         317000         317000         317000 <t< td=""><td>24.06542 33 813 19.79765 34 982 15.55703 36 246 14.63457 40 908 31.08588 41 1714 23.7671 42 985 20.51874 44 1123 40.55771 45 343</td><td></td><td>305</td><td>51804</td><td></td><td>27 40074</td><td>4</td><td></td><td>: 9</td><td>2</td><td></td></t<>	24.06542 33 813 19.79765 34 982 15.55703 36 246 14.63457 40 908 31.08588 41 1714 23.7671 42 985 20.51874 44 1123 40.55771 45 343		305	51804		27 40074	4		: 9	2	
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2.02         1.1.2.1.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.	54. 55.570.2         44.750.2         55.570.2         55.570.2         44.750.2         55.570.2         45.550.2         55.570.2         45.550.2         55.570.2         45.570.2	13.79703 34 202 12.24627 38 246 14.63457 40 908 31.08588 41 1714 23.7671 42 985 20.51874 44 1123 40.55771 45 343 17.70592 47 736		200	2015		22 00842	} <b>4</b>		2 9	9 5	
2.47         1.2.24677         3.8         2.64         3.802.31         3.8         2.64         3.802.31         3.8         3.64         3.802.31         3.8         3.64         3.802.31         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.8         3.64         3.64         4.0         3.8         3.64         3.64         4.0         3.65         3.64         4.0         3.65         3.64         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.64         4.0         3.65         3.04         3.65         3.7         3.1         3.1	156 12,050/13         35 0,050/13         35 0,050/13         35 0,050/13         35 0,050/13         35 0,050/13         35 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         37 0,050/13         41 0,050/13	10.257/U3 30 626 14.663457 40 908 31.08588 41 1714 23.7671 42 985 20.9977 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736		202	.4327.0		23.89012	ę ę	·	b u	? ;	
12.24627         38         24.8 bit	1555         12.2827         40         355         12.18267         41         355         12.18267         41         355         12.18267         41         355         12.18267         41         355         12.28267         41         371         11.18367         42         355         27.18269         43         371         11.18367         44         17.74         44         17.74         44         47.75         37.25         42.86896         56         371         11.18367         44         17.74         44         17.74         44         44         47.74         44         47.74         44         47.74         44         47.74         44	12.24627 38 246 14.63457 40 908 23.7671 42 965 22.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736		8	00400		00.4000	₽ Z		0. 9	÷ (	
146 2467   41 248 25   41 248 25   41 248 25   41 248 25   42 24 268 24 248 25   42 24 268 24 24 28 23 24 24 28 24 24 28 23 24 24 28 24 24 28 24 24 28 24 24 28 24 24 28 24 24 28 24 24 28 24 24 28 24 24 28 24 24 24 24 24 24 24 24 24 24 24 24 24	156   14.55457   4.1   4.14   4.5.6958   4.1   3.5.6   3.10   3.1.5050   4.2   3.1.5050	14.63457 40 908 31.08588 41 1714 22.37671 42 985 20.51874 44 1123 40.55771 45 343 17.70592 47 736		cge Se	.19656		24.04203	តិដី		2 9	<del>2</del>	
799         31,08888         41         1714 A46885         42         430,08888         41         1714 A56885         42         430,08888         41         171,13000         43         30,310888         41         171,13000         43         30,310888         41         41,21,13000         43         30,310888         41         41,21,13000         43         41         779         31,1373         45         324,16885         66         41         71,13100         44         779         31,1372         45         324,16885         66         32,16485         47         41         41         41,133,3000         41         51,1372         41         51,1372         42         30,40587         41         41,133,414         49         41         77,1372         41         51,1372         41         51,1372         41         51,1372         41         51,1372         41         51,1372         41         51,1372         41         41,1373         41         41,1373         41         41,1373         41         41,1373         41         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373         41,1373 <td>799         1108588         41         114         44,68989         42         480         23,925         43         202,43689         46         417         119         44,68989         417         114         44,68989         44         114         44,68989         44         114         44,68989         44         11,147         44,68989         46         30,24889         46         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,104,1048         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         410,104,104,104,104         48         30,248,1049         48         410,104,104,104         48         30,248,104,104         48         30,248,104,104         48         30,248,104,104         48         30,248,104         48         30,248,104         48         30,248,104</td> <td>31.08588 41 1714 23.7671 42 985 29.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736</td> <td></td> <td>336</td> <td>19264</td> <td></td> <td>33.00301</td> <td>នួះ</td> <td></td> <td>2 1</td> <td>3 :</td> <td></td>	799         1108588         41         114         44,68989         42         480         23,925         43         202,43689         46         417         119         44,68989         417         114         44,68989         44         114         44,68989         44         114         44,68989         44         11,147         44,68989         46         30,24889         46         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,1047         48         30,24889         48         410,104,1048         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         30,248,1049         48         410,104,104,104,104         48         30,248,1049         48         410,104,104,104         48         30,248,104,104         48         30,248,104,104         48         30,248,104,104         48         30,248,104         48         30,248,104         48         30,248,104	31.08588 41 1714 23.7671 42 985 29.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736		336	19264		33.00301	នួះ		2 1	3 :	
48         23,71771         42         965         417,21,87907         45           75         25,93777         42         915,307319         44         779         11,15737         46         304         19,0132         56         417,21,87907         45           75         25,99377         45         31,04747         46         304         19,0132         56         86         31,0447         48           126         44         112,33         46         130,33749         46         304         19,0132         56         86         31,0447         48           126         45,717,0622         47         32,54566         46         326,4566         47         51,17362         18,183         48         236,14760         51,17362         18,183         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         47         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48         236,14760         48	44         25         34         34         34         34         34         34         344         44         77         31         35         34         348         35         35         34         34         35         34         34         35         34         34         35         34         34         35         34         34         35         34         34         35         34         34         35         34         34         35         34         35         34         35         34         35         35         34         35         35         35         35         35         34         35         34         35         34         3	23.7671 42 985 29.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736	-	480	.92561		24.66411	ន		<u>.</u>	4 :	
756 205977         44 123 36, 91, 91, 91, 91, 91, 91, 91, 91, 91, 91	370         381         481         382         381         381         481         382         381         381         481         382         381         481         382         381         481         382 <td>20.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736</td> <td>-</td> <td>305</td> <td>93908</td> <td></td> <td>24.95889</td> <td>8 8</td> <td></td> <td><u> </u></td> <td>មិ រ</td> <td></td>	20.99797 43 915 20.51874 44 1123 40.55771 45 343 17.70592 47 736	-	305	93908		24.95889	8 8		<u> </u>	មិ រ	
7.86         7.60 <th< td=""><td>200         300         310         320         310         410         420         320         310         420         320         310         410         420         420         320         410         420</td></th<> <td>20.51874 44 1123 40.55771 45 343 17.70592 47 736</td> <td></td> <td>779</td> <td>.15737</td> <td></td> <td>21.63952</td> <td>gg :</td> <td></td> <td><u> </u></td> <td>47</td> <td></td>	200         300         310         320         310         410         420         320         310         420         320         310         410         420         420         320         410         420	20.51874 44 1123 40.55771 45 343 17.70592 47 736		779	.15737		21.63952	gg :		<u> </u>	47	
1288         40.55771         45         343         20.56274         47         756         33.56468         47         751         55.23256         64         322         31.4452         51.4452<	1286         405 5771         45         343         20.56274         47         541         25.23230         64         32.21,37741         48         246         25.23230         64         32.23230         64         32.2330         64         32.2330         64         32.2330         67         32.2330         67         32.2330         48         246         16.9173         49         82.3         31.2330         Min         26         17.35723         67         32.330         67         32.330         67         32.330         67         32.330         67         32.330         67         32.347803	40.55771 45 343 17.70592 47 736		1301	.77202		19.0132	S		2	48	
270         17.75592         47         736         29.52664         48         246         16.91179         48         823         31.3452         Sitats         Area         Dimeter (mean)         50           147         12.24332         48         1019         35.03419         49         326         14.8813         49         30.96987         Min         26         17.3729         16.6897         Min         17.3729	70         17.70592         47         756         29.52664         48         246         16.9179         48         823         31.3452         Min         256         17.3729         50         222           147         12.0852         48         401         35.0643         48         32         13.48613         61         77         29.21987         Min         256         17.37729         70         20.0000         70         70         20.0000         70	17.70592 47 736	•	929	56456		25.22305	9		=	<b>4</b>	
147         12.2332         48         1019         35.03419         49         326         19.43613         49         802         30.88987         Min         256         17.35729         561         17.23322         48         20.3419         49         326         19.43613         49         326         19.43613         57         722         20.21968         (Obj.#)         30	4/8         1019         35.03419         4/9         326         19.43613         4/9         320         30.20.88897         Min         266         17.3729         61         30         30         30         51         47.22436         61         48         48         326         19.43613         51         727         20.208897         Min         206         30         30         30         51         48         48         30         30         30         30         51         48         48         48         20         48 <td></td> <td></td> <td>246</td> <td>91179</td> <td></td> <td>31.3452</td> <td></td> <td></td> <td>r (mean)</td> <td>20</td> <td></td>			246	91179		31.3452			r (mean)	20	
196   14,8891   50   634   27,07093   52   774   30,4052   53   347803   55   3	96 14.88913         49 487 23.96436         52 774 30.40629         51 727 29.21968         70 12.9198	12 24352		326	43613		30.86987	Z.	17.357	g,	51	
254         6.8933         5.0         6.4         27.07033         5.3         5.3         5.3         5.3         5.4         2.80         7.65         2.9,4201         Max         2.162         5.4,34785         5.4         2.86         1.1605         Max         2.165         2.9,4201         Max         2.182         4.3,4785         5.4         2.96         1.1605         Min         2.1         2.2         4.1,54865         5.4         2.2         1.1605         Min         2.2         1.152         4.1,54865         5.4         2.2         1.1605         Min         1.0 </td <td>254         16.88913         50         634         27.07093         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         54         284         110         0.01,#*         45         45         45         45         45         44         45         45         45         45         45         45         45         45         45         45         45         45         45         45         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44</td> <td>14 86915 49 487</td> <td></td> <td>774</td> <td>40629</td> <td></td> <td>29.21968</td> <td>(Opj:#)</td> <td></td> <td>2</td> <td></td> <td></td>	254         16.88913         50         634         27.07093         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         53         33.47803         54         284         110         0.01,#*         45         45         45         45         45         44         45         45         45         45         45         45         45         45         45         45         45         45         45         45         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44         45         44	14 86915 49 487		774	40629		29.21968	(Opj:#)		2		
604         65         51         1322         41.54865         54         286         1456         Ann         230         156         287.86         51         1322         41.54865         57         238         16.5477         Min         230         16.6882         Range         142         330         430         230         16.6882         Range         1432         38.9036         58.9036 <td>  State   Stat</td> <td>16 86913 50 634</td> <td></td> <td>335</td> <td></td> <td>52 765</td> <td>29.94201</td> <td>Max</td> <td>8.34</td> <td></td> <td>Ē</td> <td>15.025</td>	State   Stat	16 86913 50 634		335		52 765	29.94201	Max	8.34		Ē	15.025
State   Stat	2012         2013         1322         1324 <th< td=""><td>20 00 00 00 00 00 00 00 00 00 00 00 00 0</td><td></td><td>900</td><td></td><td>Area</td><td>Diameter (mean)</td><td>(Opj:#)</td><td></td><td></td><td></td><td></td></th<>	20 00 00 00 00 00 00 00 00 00 00 00 00 0		900		Area	Diameter (mean)	(Opj:#)				
528 27.9984 53 472.27933 58 420 10.04ff) 10 10 Mean 754.9474 28.71915 58 20.11852	SST 200112         SST 200114         SST 200	26.09/30 51		200			16.5682					
281 17.5321 Stats Area Diameter (mean) 59 1082 71.8954 States Area Diameter (mean) 59 1082 71.8954 States Area Diameter (mean) 59 1082 71.8954 States Area Diameter (mean) 5144 States Area Diameter (mean) 614.83235 Min 190 14.83235 Mean 653.0952 27.45478 Samples 38 38 38 58 16.65573 Min 190 10.25018 States Area Diameter (mean) Mean 782.7273 29.47727 Range 138 2.3 Samples 4 4 4 4 4 4 8 Samples 4 5 45 45 45 45 45 45 45 45 45 45 45 45	State   Stat	20,00112 02 1140		207	9		9					
221 (1-5.52.1) State Area Unameter (intent)	291 17:53221         State Area         Unameter (mean)         39 1092 3 (1094)         23 23         Sum 2868 1091.29         Mean 667.125           223 6:56233         Min         196 14.60063         State Area         Diameter (in Range 1415 27.9272         Samples 38         38 38         Std.Dev 519.71 9.           524 6:56334         Min         190 14.8325         Mas 63.0952         27.45476         Samples 38         38 38         Std.Dev 519.71 9.           576 25.9413         Max 177829         (Obj.#)         3 0 30         Min         190 14.8325         Mas 63.0952         27.45476         Samples 38         38 38         Std.Dev 519.71         Std.Dev 51.7629         Samples 40.8520         Samples 40.8520         A2 42         A	1.59886.12		074			44.49541		0,	ž.		ĕ
232         16 682/3 Min         Min         195 14.90053         Stats Area         Usiminater (I         Range         1415 27.9272         Samples         38 </td <td>232         16 662/3 (b) (b)         Min         196 14 60063         State Area         Usample (f) (Range         Range         1415 27 9272         Samples         38         Std. Dev         519,721         98 109           524         42,8019         (Obj.#)         7         7         Man         163.062         27,45476         Samples         38         38         Std. Dev         519,721         98 108           296         18,77829         (Obj.#)         30         30         Max         1570         43.80937         Sum         27,234         6,139822         Smples         40           299         18,77829         (Obj.#)         30         30         Max         1570         43.80937         Sum Page         42         42         42         42         40         10         10         10         10         10         11         23         14         42         <td< td=""><td>17.53221 Stats Area</td><td>i</td><td>1082</td><td>۳</td><td></td><td>23</td><td>Sum</td><td></td><td><b>6</b></td><td></td><td></td></td<></td>	232         16 662/3 (b) (b)         Min         196 14 60063         State Area         Usample (f) (Range         Range         1415 27 9272         Samples         38         Std. Dev         519,721         98 109           524         42,8019         (Obj.#)         7         7         Man         163.062         27,45476         Samples         38         38         Std. Dev         519,721         98 108           296         18,77829         (Obj.#)         30         30         Max         1570         43.80937         Sum         27,234         6,139822         Smples         40           299         18,77829         (Obj.#)         30         30         Max         1570         43.80937         Sum Page         42         42         42         42         40         10         10         10         10         10         11         23         14         42 <td< td=""><td>17.53221 Stats Area</td><td>i</td><td>1082</td><td>۳</td><td></td><td>23</td><td>Sum</td><td></td><td><b>6</b></td><td></td><td></td></td<>	17.53221 Stats Area	i	1082	۳		23	Sum		<b>6</b>		
524         24,8019         (Obj.#)         32         16         Min         190         14,83235         Mean         653.0952         27,45476           256         25.9413         Max         173         45,71595         (Obj.#)         7         7 Std.Dev         277,2394         6,139822           298         18,77829         (Abj.#)         30         Max         1570         43,80937         Sum         277,2394         6,139822           229         16,27969         Range         1534         31,11533         (Obj.#)         32         Samples         42         42           100         10,25018         Std.Dev         477,727         Range         1390         28,97702         A2         42           9         Sum         3440         1297         Std.Dev         369,8065         8,007271         A5           1185         30,30754         Samples         44         44         Sum         30218         1231,154           4.55         65         65         65         65         65         45	524         24,8019         (Obj.#)         32         16         Min         190         14.83235         Mean         653.0952         27.43476         Sum         26685         1060           576         25.94113         Max         1730         45.71595         (Obj.#)         7         71.00 by         277.2346         139822         Samples         40           276         25.94113         Max         1750         43.80937         Sum         277.234         6.139822         Samples         40           229         16.27969         Range         1534         31.11533         Obj.#)         32         32         Samples         42         42         42           100         10.25018         Std. Dev         447.8669         9.309393         Mean         671.511         27.3869         65         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9         8         9 </td <td>16.66273 Min 196 14.6000</td> <td></td> <td></td> <td></td> <td>_</td> <td>27.9272</td> <td>Samples</td> <td></td> <td>88</td> <td></td> <td></td>	16.66273 Min 196 14.6000				_	27.9272	Samples		88		
576         25.94113         Max         1730         45.71595         (Obj.#)         7         Std. Dev         277.2394         6.139822           229         18.77829         (Obj.#)         30         Max         1570         43.80937         Sum         27430         1433.1           229         18.77829         16.27969         16.27969         1534         31.11533         (Obj.#)         32         32         Samples         42         42           100         10.25018         Std. Dev         47.727         Renge         1380         28.97702         38.9065         8.007271         39.8066         8.007271         44         Std. Dev         39.8066         8.007271         45	576         25.94113         Max         1730         45.71595         (Obj.#)         7         7         Std. Dev         277.2394         6.139622         Samples         40           298         18.77829         (Obj.#)         30         Max         1570         43.80937         Sum         27.430         163.13         A2         28         163.1         28.97702         A2         28.97702         A2         <	24.8019 (Obj.#) 32					27.45476					
298 18,77829 (Obj.#) 30 30 100 Max 1570 43,80937 Sum 27430 115; 229 18,27969 Range 1534 31,11533 (Obj.#) 32 Samples 42 10,115,25018 Std.Dev 447,8669 9,309393 Mean 671,5111 27,35898 Std.Dev 447,8669 9,309393 Mean 671,5111 27,35898 Std.Dev 26,5771 Samples 44 44 Sum 30218 1231,154 Samples 45 65 65 65 65 700271 Samples 45 44 44 Samples 45 21,70677	298 18,77829 (Obj.#) 30 Max 1570 43,80937 Sum 27430 115. 229 16,27969 Range 1390 (Obj.#) 32 Samples 42 Dameter (nean) Mean 782,7273 29,47727 Range 1390 28,97702 100 10,25018 Std.Dev 447,8669 9,309393 Mean 671,511 27,35898 9 9 Sum 34440 1297 Std.Dev 369,8065 8,007271 1285 40,55771 Samples 44 44 Sum 30218 1231,154 65 65 65 1485 30,30754 44.66 21,70607	25.94113 Max 1730	9			27	6.139822				Samples	
229 16.27969 Range 1534 31.11533 (Obj.#) 32 Samples 42 Diameter (mean) Mean 782.7273 29.47727 Range 1380 29.7702 100 10.25018 Std.Dev 447.8669 9.309393 Mean 671.5111 27.35998 9 Sum 3440 1297 Std.Dev 369.8065 8.007271 1285 40.55771 Samples 44 44 Sum 30218 1231.154 65 65 65 Samples 45 45 A5 1185 30.30754	229 16.27969 Range 1534 31.11533 (Obj.#) 32 Samples 42 Diameter (mean) Mean 782.7273 29.47727 Range 1380 28.97702 100 10.25018 Std.Dev 447.8669 9.309393 Mean 671.5111 27.35898 9 Sum 3440 1297 Std.Dev 369.8065 8.007271 1285 40.55771 Samples 44 44 Sum 30218 1231.154 65 65 65 45 1185 30.30754 44 545 21.70607	18.77829 (Obj.#) 30					1153.1					
Diameter (mean)   Mean   782,7273   29,47727   Range   1380	Diameter (mean)         Mean         782,7273         29,47727         Range         1380           100         10.25018         Std.Dev         447,8669         9.309393         Mean         67,111           9         Stm         3,2440         1297         Std.Dev         369,8065           1285         40.55771         Samples         44         44         Sum         30218           65         65         65         65         8         Samples         45           4456         21.70607         36,597242         4732         6,597242         4732	16.27969 Range 1534		32		_	42					
10.25018 Std.Dev 447.8659 9.309393 Mean 871.5111 9 Sum 34440 1297 Std.Dev 365.8065 40.55771 Samples 44 44 Sum 30218 65 30.30754 Samples 45	10.25018 Std.Dev 447.8669 9.309393 Mean 871.5111 9 Sum 3440 1297 Std.Dev 369.8065 40.55771 Samples 44 44 Sum 30218 65 30.30754 Samples 45 21.70607 44 Samples 45 22.70607	Diameter (mean) Mean 782.7273		1380	.97702							
9 Sum 3440 1297 Std.Dev 369.8065 40.55771 Samples 44 44 Sum 30218 65 Samples 45 30.30754 Samples 45	9 Sum 3440 1297 Std.Dev 369.8065 40.55771 Samples 44 44 Sum 30218 65 Samples 45 21.70607 6.597242	10.25018 Std.Dev 447.8669		671.5111	.35898							
40.55771 Samples 44 44 Sum 30218 1231.1 65 Samples 45 30.30754 45 21.70607	40.55771 Samples 44 44 Sum 30218 1231.1 65 Samples 45 Samples 45 21.70607 8.597242	9 Sum 34440		369.8065	007271							
65 Samples 45 30.30754 45 21.70607	65 Samples 45 30.30754 21.70607 6.597242	40.55771 Samples 44		30218	31.154							
30.30754 21.70607	30.30754 21.70607 6.597242	65 65	Sample		5							
			,									

# Chart 1: Colony forming efficiency test for diff. Doses of Ethanol.

0 1	Obj.# Area Diameter (I	1 981	98	3 627 29.62269		Ţ		1074	•		10 671 28.0817	11 956 34.29499	12 977 34.44915	•	208	1547	452	378	1376	612	284 1	2120	874 3	2446	190	1243	1324	29 489 23.73582	266	398	37 904 34,10687	38 1326 40.3833	39 891 33.26693	Stats Area Diameter (i	208 15.605	4	Max 2446 54.73	24		928.9394	520.7398	30655 1069.1	Samples 33 33	
•	Diameter (mean)	18.84619	19.13212	47.4571	19.42469	20.03454	53.89959	19.38202	60.42813	36.68781	28.04055	20.92064	56.46736	19.39491	38.13656	38.62394	23.73361	39.52578	25.93602	27.13687	24.28621	57.31591	24.75276	30.94194	19.49257	16.84583	27.60254	20.67114 24 85555	21 99871	17,76215	27.06714	68.06725	34.38712	29.96356	49.61019	46.23339	Diameter (mean)	16.84583	36	68.06725	46	51.22141	32.00173	13.99589
	Area	311	318	1861	319	338	2393	319	2921	1095	899	377	2657	314	1229	1235	470	1179	564	637	499	2715	521	808	320	243	591	350	1 4 1 C	278	620	3746	981	757	2014	1735		243	36	3746	46	3503	1005.472	893.8641
ı	Plate / Obj.#	-	· 4	က	4	ဖ	7	ω	თ	10	12	13	15	18	7	55	23	24	25	27	58	30	3	35	35	98	38	30	4 4	4	45	46	47	48	4	20	Stats	Μij	(Opj.#)	Max	(Obj.#)	Range	Mean	Std.Dev

Fig. 1: Colony forming efficiency test for diff. Doses of Ethanol.

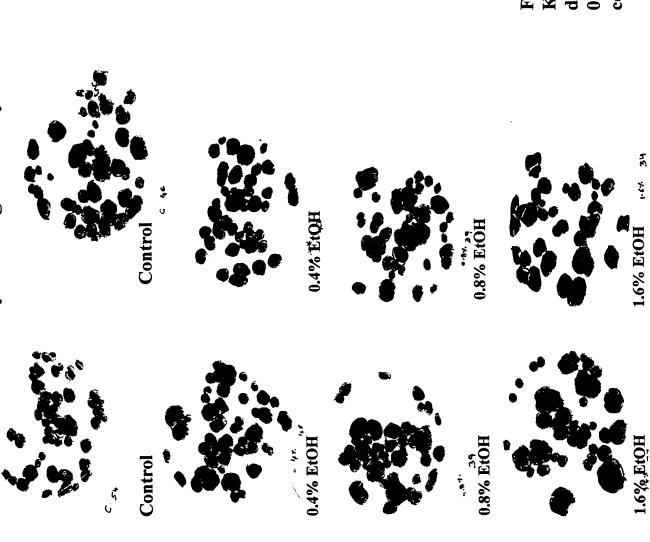
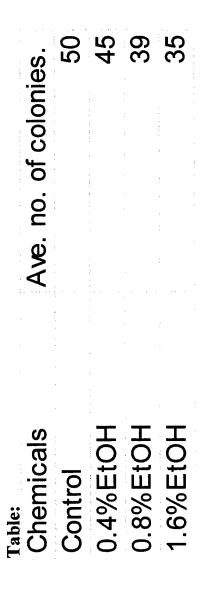


Fig. 1: Colonies shown by the Keratinocytes after the exposure to different doses of ethanol (0.4%, 0.8%, 1.6%) along with untreated control

Fig.2: Graphical representation of colony forming efficiency of keratinocytes exposed to diff.doses of ethanol.



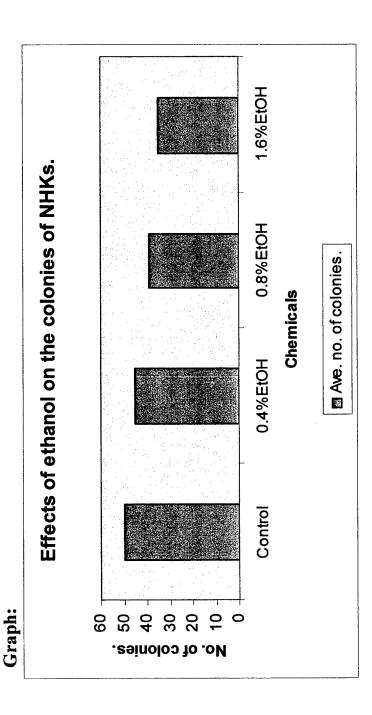
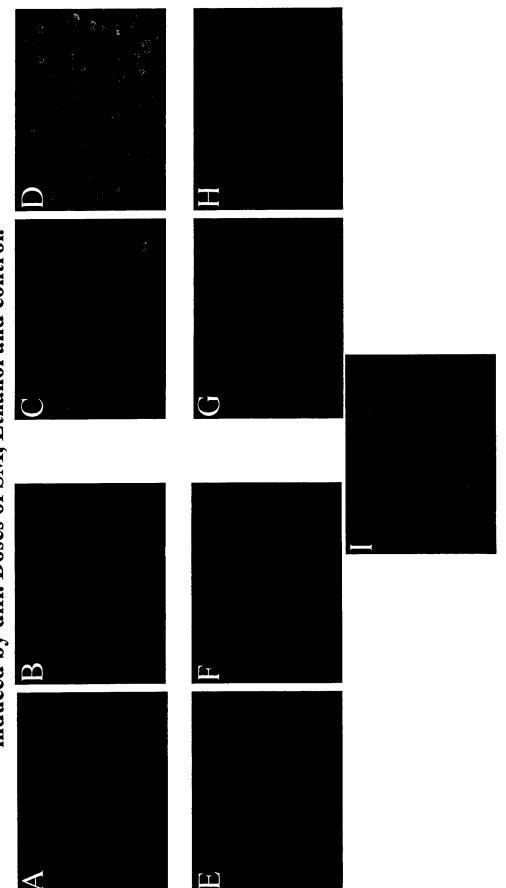


Fig. 3: Immunofluorescent staining for Apoptotic cells induced by diff. Doses of SM, Ethanol and control.

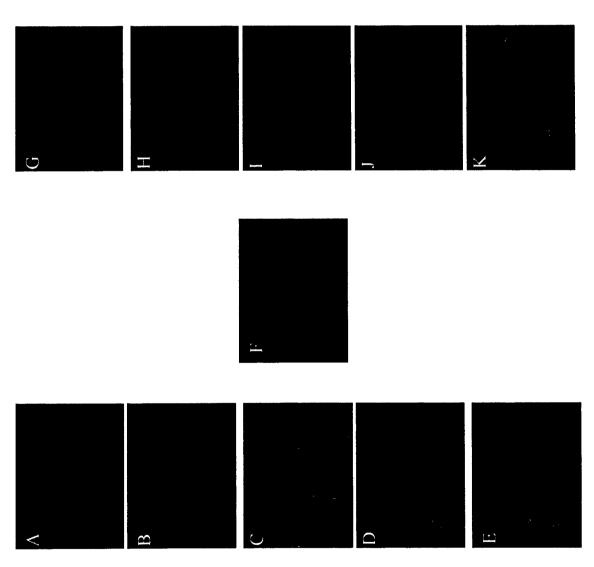


A-D -Varying doses of SM (37.5, 75, 150, 300um)

E-H – Varying doses of concurrent Ethanol controls (0.25, 0.5, 1, 2%)

I – Untreated control.

Fig. 4: M30 Cytodeath staining for Keratinocytes exposed SM and Ethanol at diff. Sample intervals.

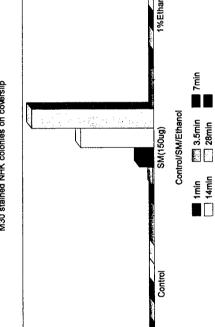


**A-E**-1, 3, 7, 14, 28 min. of 150um of SM; **G-K**-1, 3, 7, 14, 28 min of 1% Ethanol; F- Untreated control.

Fig. 5: Apoptotic cell counts (NHK)

Chemicals	1min.	3.5min.	7min.	14min.	28min.	
Control		4	4	4	4	4
SM (150u <sup>24</sup> )		18	69	795	4015	6783
1%EtOH		ဖ	13	15	18	221





1000

3000

Cell counts

2000

4000

5000

0009

### Apoptotic Cell counts

Control, SM, Ethanol (Comparision)

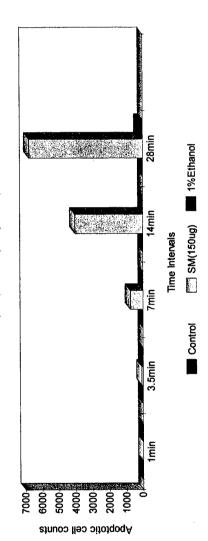


Fig. 6: M30 Cytodeath staining for 150um SM and 1% Ethanol at two different intervals.

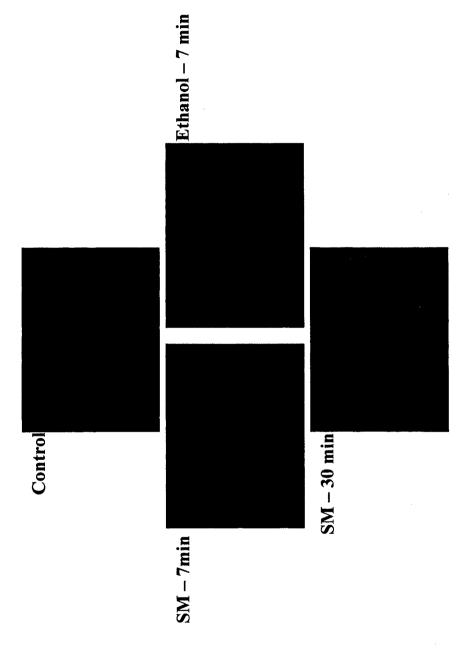


Fig.7: MTT Assay for NHK's on Plastic plates at diff. Cell density.

Plastic				
Chemica	20000	25000	10000	1000
SM	0.605	0.153	0.302	0.029
Ethanol	0.828	0.449	0.289	0.013

### MTT Assay for NHKs on Plastic plate (24

SM (150µm. for 7min); 1% Ethanol for

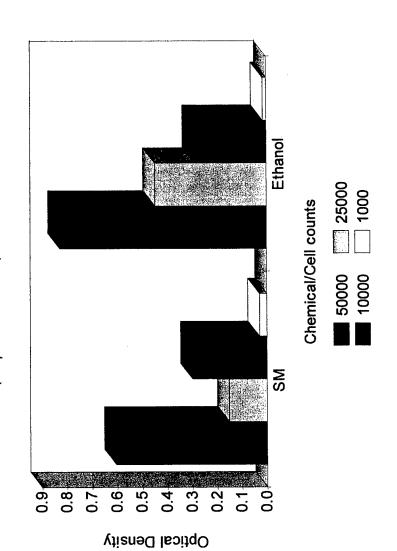
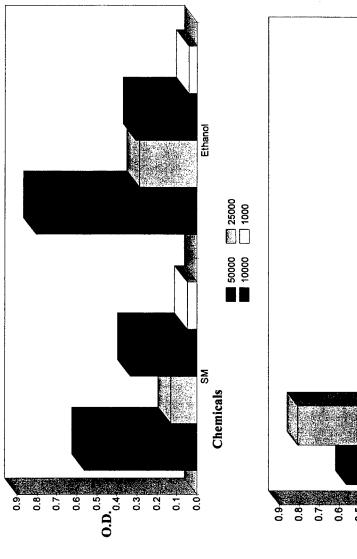


Fig. 8: MTT Assay for NHK's at varied Cell density on Type IVCollagen plates.

Chemica	20000	25000	10000	1000
SM	0.564	0.133	0.332	0.047
Ethanol	0.807	0.287	0.307	0.0413



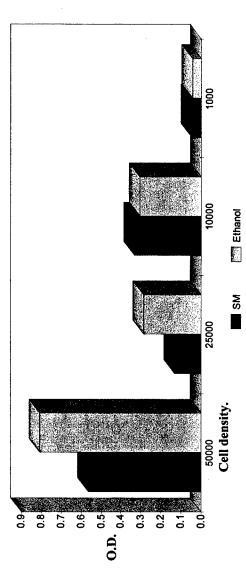


Fig. 9: MTT Assay at two different cell densities of NHK's on Col I

Chemica	20000	25000
SM	0.491	0.272
Ethanol	0.657	0.369

MTT Assay for Coll
SM and Ethanol at Cell density

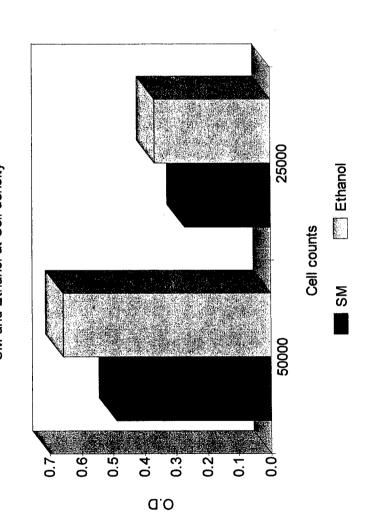


Fig.10: MTT Assay for 50000NHKs on diff. Plates.

Table:

	Plastic	ColI	Sol IV	ESN Ne	Laminin	Poly-D
SM	0.27	0.261	0.226	0.165	0.189	960.0
Ethanol	0.317	0.349	0.436	0.301	0.285	0.107

MTT assay for NHKs on different plates

Comparisions between SM and Ethanol

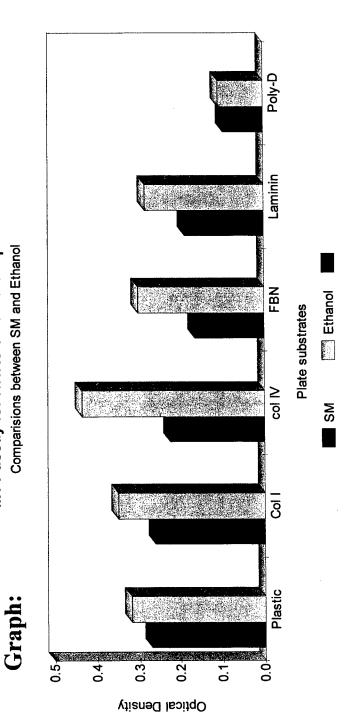
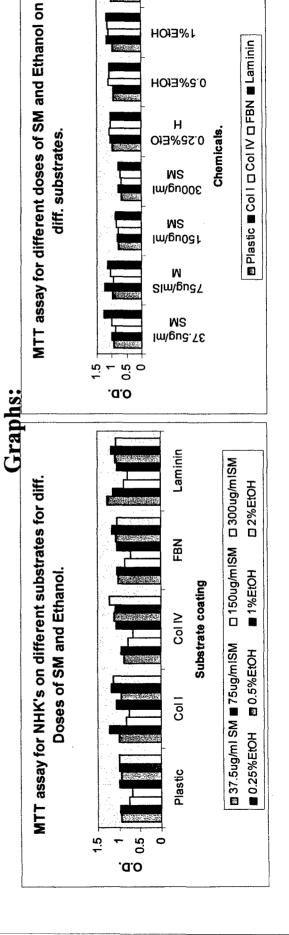


Fig. 11: MTT Assay for different doses of SM and ethanol on diff. substrates.

### Table:

Doses/coatin <sub>ξ</sub> Plastic	Coll	Coli	H N N	Laminin	Ë
37.5uming of SM	0.942	1.002	0.886	1.005	1.277
75um of SM	0.98	1.225	0.95	1.032	1.133
150u <sub>M</sub> of 3 SM	0.768	0.822	0.795	0.85	0.873
300u w of . SM	0.701	0.769	0.679	0.71	0.786
0.25% EtOH	0.991	1.059	1.067	1.038	1.044
0.5% ЕtОН	0.957	0.951	1.103	1.06	1.083
1% Еtон	0.992	1.178	1.096	1.155	1.173
2% EtOH	966.0	1.123	1.228	1.048	1.068

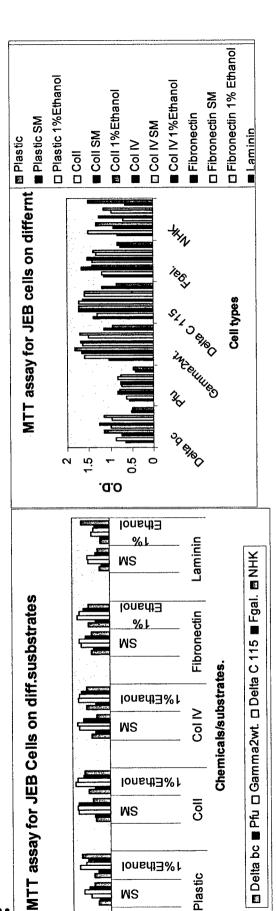


**2%EtOH** 

1%ЕЮН

Fig. 12: MTT assay for JEB Cells on diff. Substrates. (150um SM and 1% Table: ethanol)

(	I able:								
	S	strates.	ubstrates. Delta bc	Pfu	Ga	mma2wt. De	Gamma2wt. Delta C 115 Fgal.		NHK
	Plastic								
	SM		0.649		0.572	1.03	1.396	1.152	0.842
	1%	1%Ethanol	0.868	0.6	0.638	1.601	1.3	1.191	1.512
	Coll								
	SM		0.749		0.836	1.673	1.74	1.674	0.931
	1%	%Ethanol	1.153	0.	0.797	1.823	1.724	1.408	1.327
	Col ≥								
	SM		0.996		0.75	1.645	1.73	1.416	0.687
	1%	l%Ethanol	1.253		0.765	1.676	1.639	1.531	1.268
	Fibronectin								
	SM		0.97	õ	0.842	1.497	1.599	1.318	0.973
	1%	1% Ethanol	1.143		0.757	1.707	1.595	1.402	1.149
	Laminin								
	SM		0.526		0.418	1.141	1.198	0.766	0.658
	1%	1% Ethanol	0.494		0.475	0.954	0.852	0.833	1.499



WS

WS

WS

WS

.0.0 .4.0 .4.0

1%Ethanol

1%Ethanol

1%Ethanol

Chemicals/substrates.

<u>Col</u> ≤

<del></del>8

Plastic

Fig. 13: MTT Assay for diff. Cell types on diff. Substrates.(300um SM & 2% Ethanol.

Table: Diggie		Del bc Pfu		Gam2wt	Gam2wt Del C115	fgal	NHK	¥
	SM	0.804	0.489	0.744	0.682		1.008	0.875
	Ethanol	0.875	0.447	0.885	0.63		1.151	1.187
Col								
•	SM	0.838	0.82	1.064	1.196		1.034	0.751
•	Ethanol	0.956	0.968	1.219			1.429	1.263
≥ Ioo								
	SM	0.316	0.291	0.373	0.441	0	0.369	0.328
	Ethanol	0.356	0.373	0.419	0.469		0.465	0.369
FBN			•					
	SM	0.677	0.78	0.885	0.971	<del>-</del>	1.162	0.944
	Ethanol	0.78	0.924	1.03	1.138		1.433	1.347
Laminin								
	SM	0.606	0.359	0.658	0.738		0.823	0.935
	Ethanol	0.474	0.285	0.697	0.479		0.831	1.114

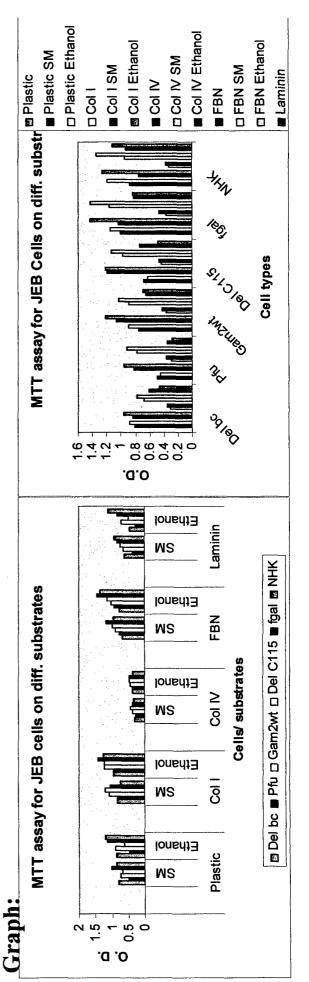


Fig. 14: MTT assay for 75um SM on diff. Substrates.

•
hla
F

Substrat	Substrates Chemicals De	Delta bc Pfu	•	Gam. Wt Do	Delta C11t Fgal	<del>_</del>	ZTZ
<u>-</u> 8	SM(75u <sub>23</sub> )	1.433	1.219	2.021	1.376	1.421	1.265
	0.5% EtOP	1.549	1.216	2.064	1.474	1.664	1.527
Col≤	SM(75u <sub>26</sub> )	1.372	0.992	1.851	1.414	1.459	1.392
	0.5%EtOH	1.33	1.039	1.99	1.318	1.448	1.512
FBN	SM(75um)	0.979	0.828	1.606	1.089	1.257	0.968
	0.5%EtOH	1.201	0.997	1.898	1.184	1.306	1.193

Graph:

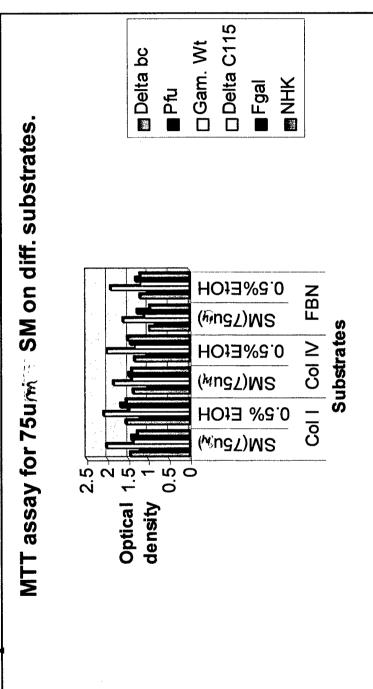


Fig. 15: MTT assay for 150um SM on diff. Substrates.

Table:						
Substrate	es Chem.		Pfu	Gam. Wt	Delta C11f Fgal	al NTK
Col	Coll SM (150u™ 1.192	1.192	0.954	1.633	1.349	1.115
	1% EtOH	1.288	1.156	2.095	1.315	1.455
<u>Col</u>  ≤	SM (150ug	1.126	1.094	1.59	1.134	1.258
	1%EtOH	1.145	0.98	1.836	1.238	1.264
FBN	SM (150um	٥.907	0.739	1.627	1.158	1.068
	1% EtOH	1.169	0.985	1.816	1.074	1.041

1.116 1.309 1.446 1.568 1.03

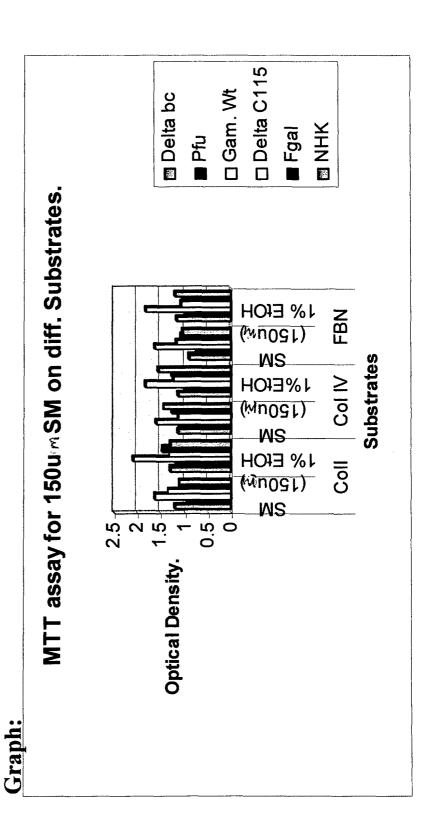
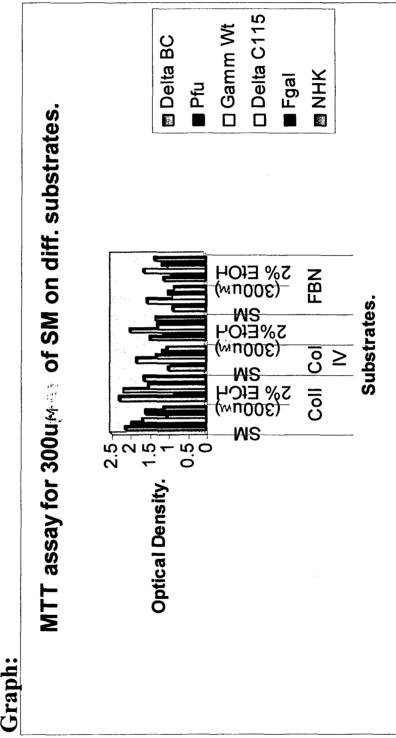


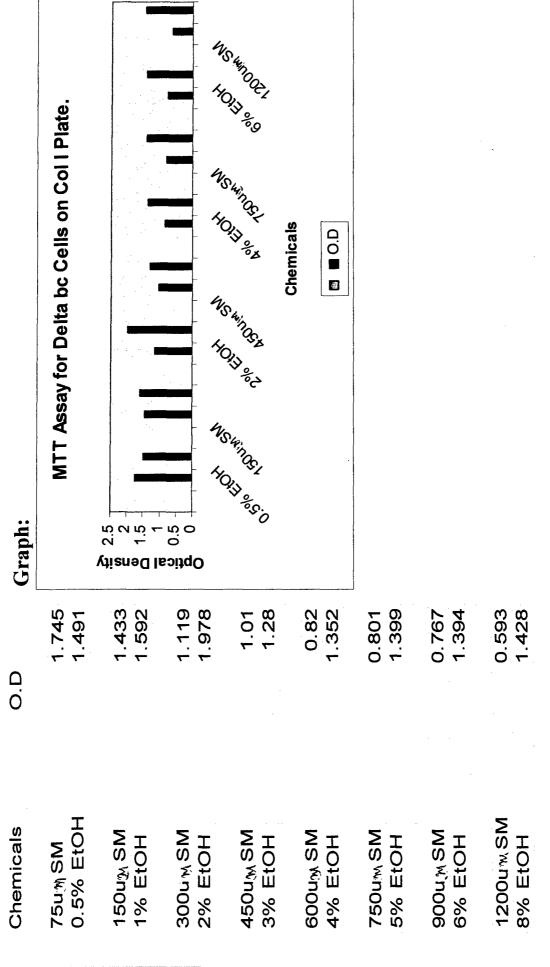
Fig. 16: MTT Assay for 300um SM on diff. Substrates.

75 Pft 88 7 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	S Delta BC Pfu 2.115 2.277 0.998 1.478 1.478
 25 88 75 P	Delta BC Pfu 2.115 2.277 0.998 1.478 0.876 1.103



# Fig. 17: MTT Assay for Delta be Cells on Col I Plate.

### Table:



### Fig. 18: MTT Assay for Delta bc Cells on Col IV Plate.

### Table:

Chemicals

0.5% EtOH 75u™ SM

150um SM **1% EtOH**  300u<sub>™</sub> SM 2% EtOH 450um SM 3% EtOH 600us SM **4% EtOH**  7500m SM **5% Etoh**  MS ™noo6 **6% EtOH**  1200um SM 8% **EtOH** 

Graph:

MTT Assay for Delta bc cells on Col IV Plate. .292 1.628

1.539 1.32

Optical density

0.5

1.103 1.168

Wormood,

MOIT SO

W. MOST

Wonings

Chemicals

0.0 ■

1.112

0.909

0.729

0.743

0.701

# Fig 19: MTT Assay for Fgal Cells on Col I Plate.

Graph:			MS HOLD MS HOLD MS HOLD MS HOLD OS					
O.D	1.49	1.317	1.202	0.979	1.078	0.884	1.003	0.82
Table: Chemicals	75um SM 0.5% EtOH	150u <sup>™</sup> SM 1% EtOH	300u ⋈ SM 2% EtOH	450u <sup>™</sup> SM 3% EtOH	600u <sup>™</sup> SM 4% EtOH	750u ⋈ SM 5% EtOH	900u <sup>™</sup> SM 6% EtOH	1200u <sup>n</sup> SM 8% EtOH

Fig. 20: MTT Assay for Fgal cells on Col IV Plate.

Graph:			WE'N HOUT OOD WE'N OOK WE'N OOK OOK OOK OOK OOK OOK OOK OOK OOK OO	Doses of SM and Ethanol □ ■ O.D.				
O.D.	1.468	1.27	0.841	0.894	0.818	0.742	0.596	0.544
Table: Chemicals	75u™ SM 0.5% EtOH	150u <sup>™</sup> SM 1% EtOH	300ug <sub>1</sub> SM 2% EtOH	450u <sub>v1</sub> SM 3% EtOH	600u™ SM 4% EtOH	750u <sub>M</sub> SM 5% EtOH	900u <sub>™</sub> SM 6% EtOH	1200um SM 8% EtOH

Fig. 21: H & E Staining for Rafts exposed to SM and Ethanol.

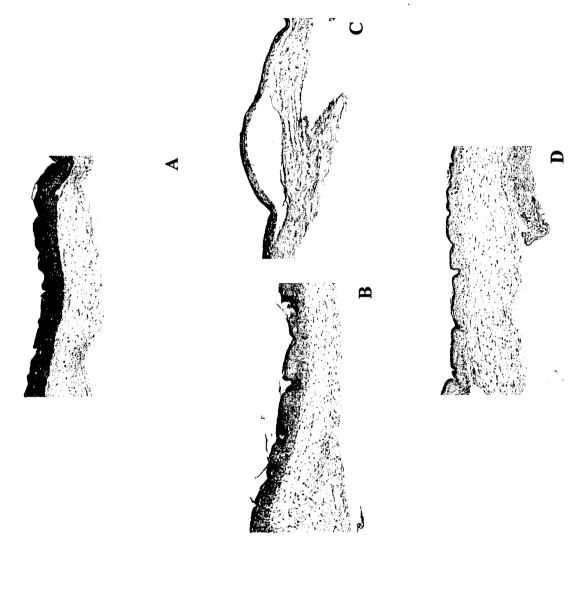


Fig: A- Control; B & C - 75 & 150um SM; D- 1% Ethanol.

Fig. 22: H&E Staining for Rafts exposed to diff. Doses of SM along with **Controls** 

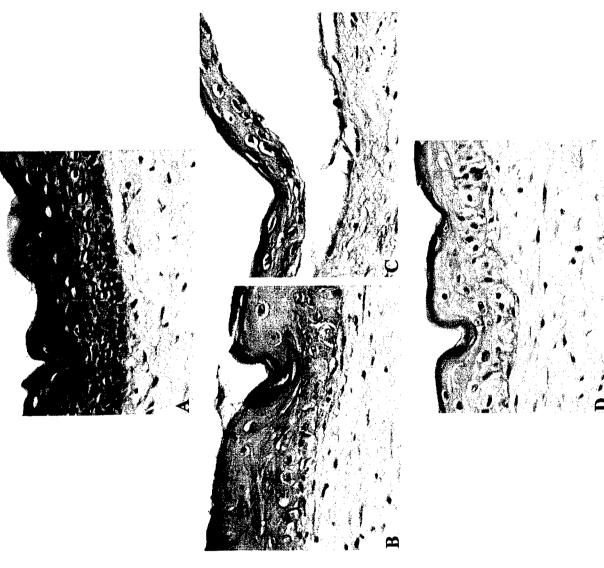
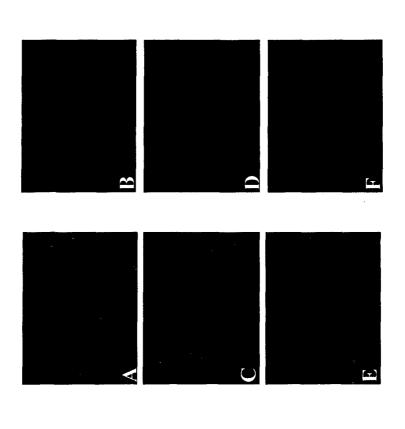


Fig: A - Control; B & C - 75 & 150 um SM; D - 1% Ethanol.

Fig. 23: M30 Staining for Rafts exposed to SM and Ethanol.



exposed to 75um SM; E& F - Raft exposed to 150um SM. Fig: A& B - Raft exposed to 1% Ethanol; C&D - Raft

Fig. 24: H&E Staining for diff. doses of SM and Ethanol controls.

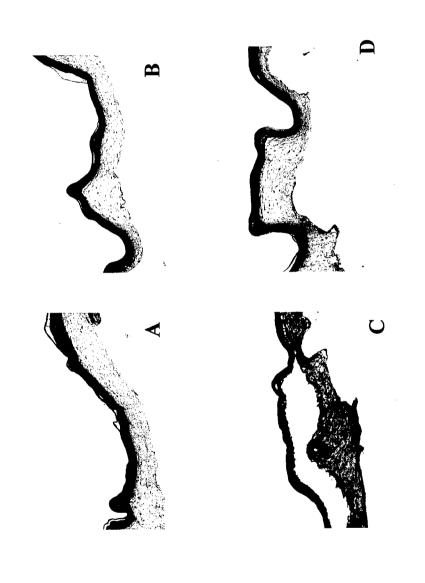


Fig: A & C-150 &300um of Sulfur Mustard.

B & D-1 & 2 % Concurrent Ethanol controls.

Fig. 25: H & E Staining for diff. Doses of SM and Ethanol.



Fig: A & C - 150 and 300um of SM;

B & C − 1 and 2 % Ethanol controls.

Fig. 26: M30 Staining for the rafts exposed to diff. doses of SM and Ethanol

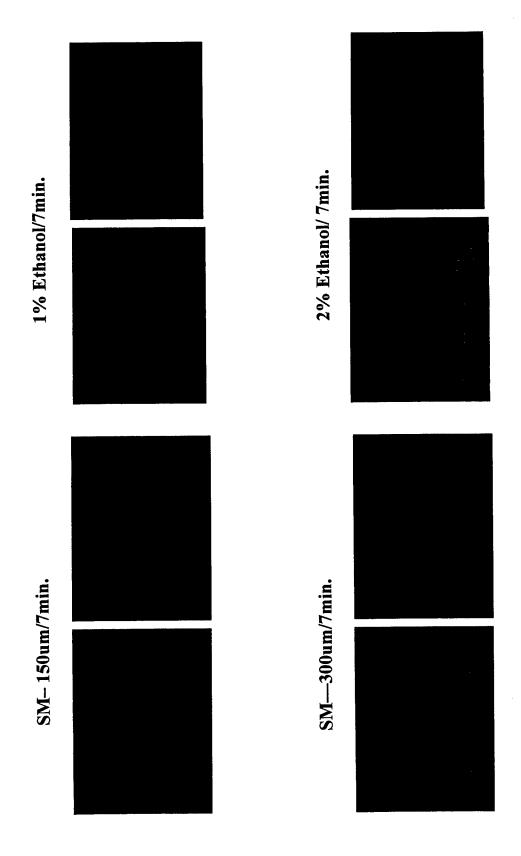


Fig. 27: H&E Staining for NHK's on different substrates (3-D 1 % Ethanol SM (150um) culture)

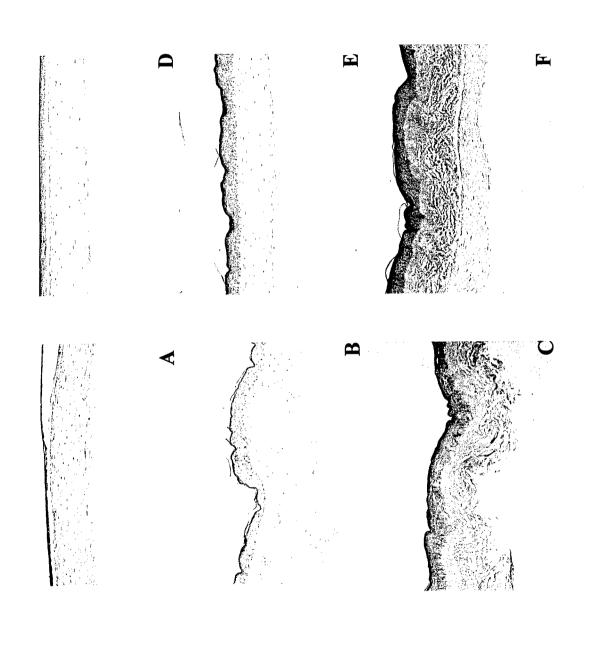


Fig: A&D- Plastic; B&E- Raft; C&F- Alloderm.

Fig. 28: H & E Staining for NHK's on diff. substrates.

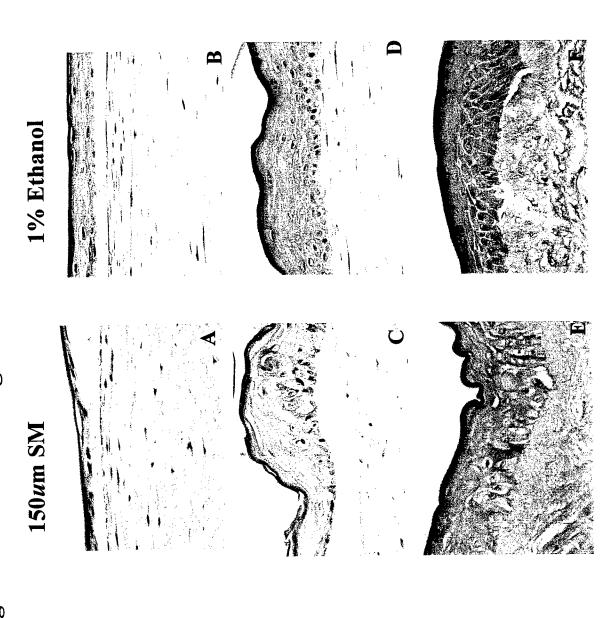


Fig: A&B - Plastic; C&D - Raft; E&F - Alloderm.

Fig. 29: H & E Staining for NHK's on diff. Substrates (3- D cultures)

SM (150um)

1% Ethanol

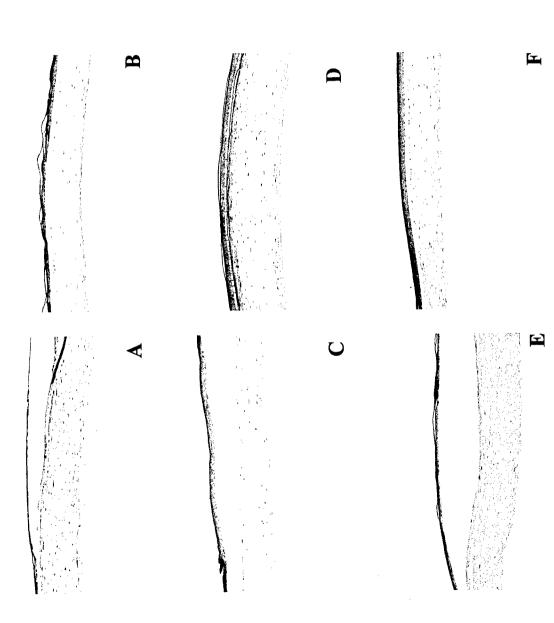


Fig: A&B- Col I; C&D- Col IV; E& F- Fibronectin.

Fig. 30; Effects of SM and Ethanol on NHK's on diff. Substrates (3-D)

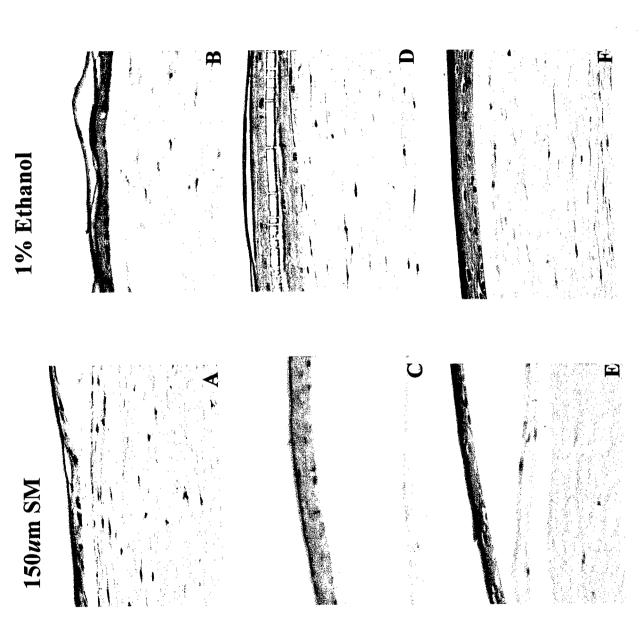


Fig: A&B - Col I; C&D - Col IV; E&F - Fibronectin.

Fig. 31: Effects of SM on Alloderm and Raft along with controls.

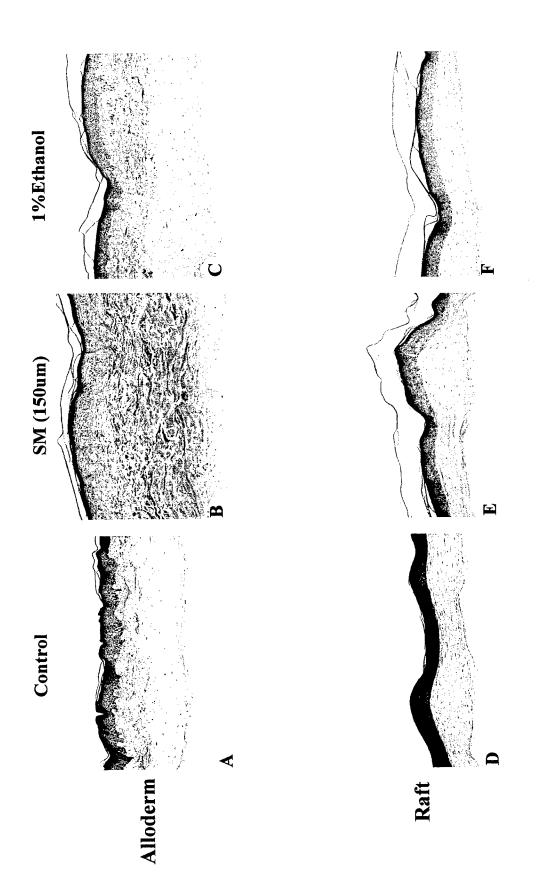


Fig: A& D - Control; B& E - 150um SM; C&F - 1% Ethanol.

Fig. 32: Effects of SM on Alloderm and Rafts along with controls.

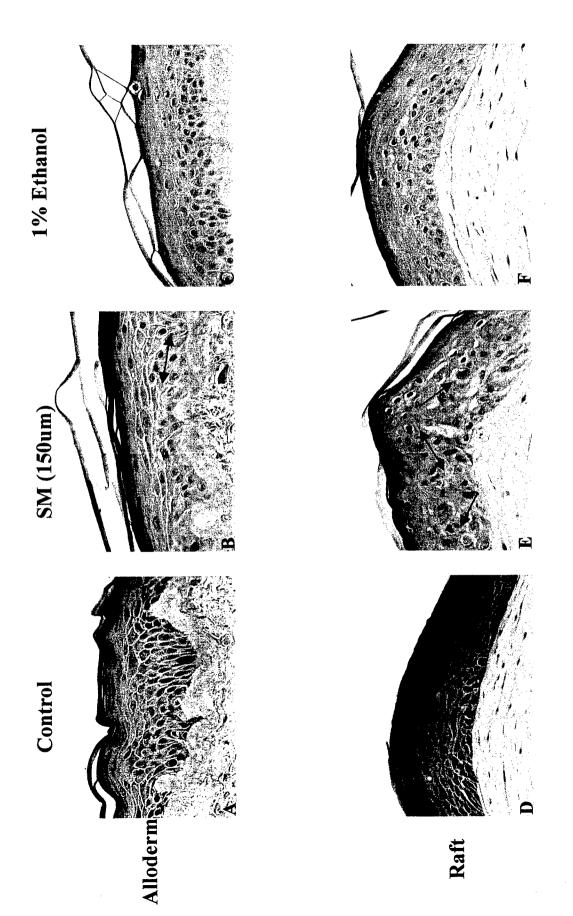
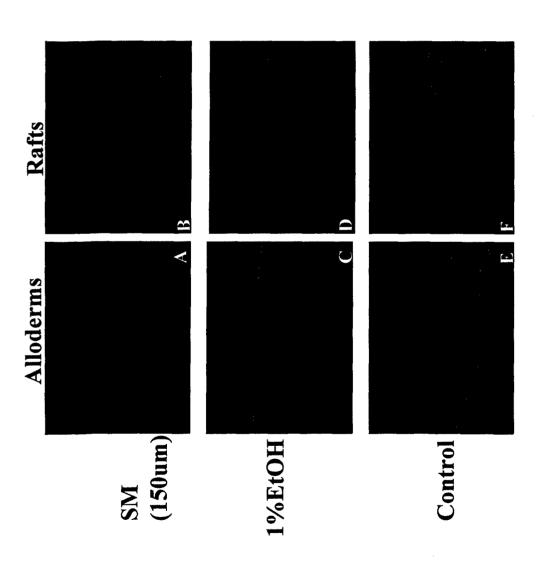


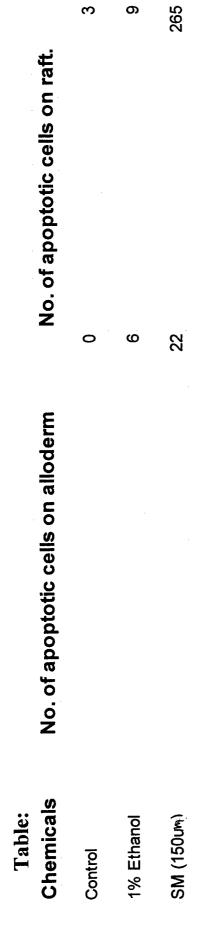
Fig: A&D - Controls; B&E - 150um of SM; C&F - 1% Ethanol.

Fig 33: M30 Staining for Alloderms and Rafts exposed to SM and Controls.



A,C, E -Alloderms; B, D, F- Rafts.

Fig. 34: Apoptotic cell counts for keratinocytes on rafts and alloderms.



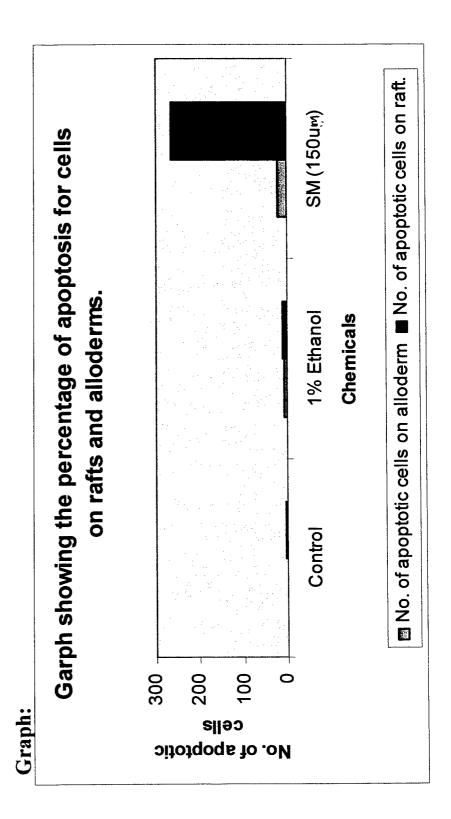


Fig. 35: Alloderm exposed to diff.doses of SM and Controls.

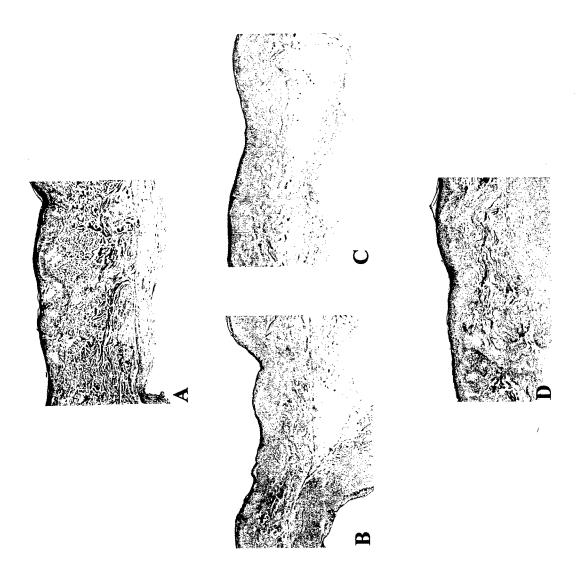


Fig: A-Control; B&C- 75& 150um SM; D- 1% Ethanol.

Fig. 36: Alloderm exposed to diff. Doses of SM and Controls



Fig: A- Control; B& C - 75 & 150um SM; D- 1% Ethanol.

Fig. 37: M 30 Staining for Alloderm exposed to SM and Controls.

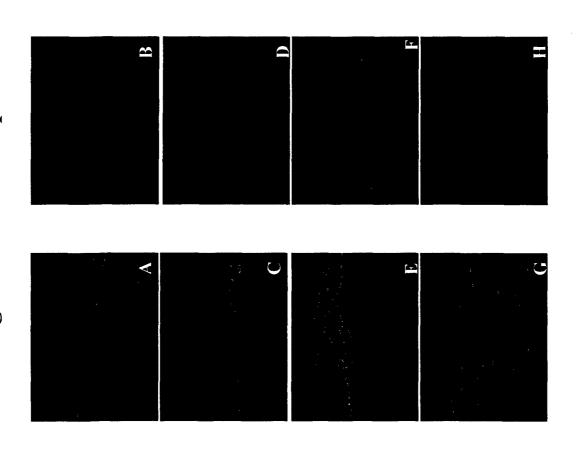


Fig: A, B- Control; C, D- 75 um SM; E, F-150um SM; G, H- 1% Ethanol.

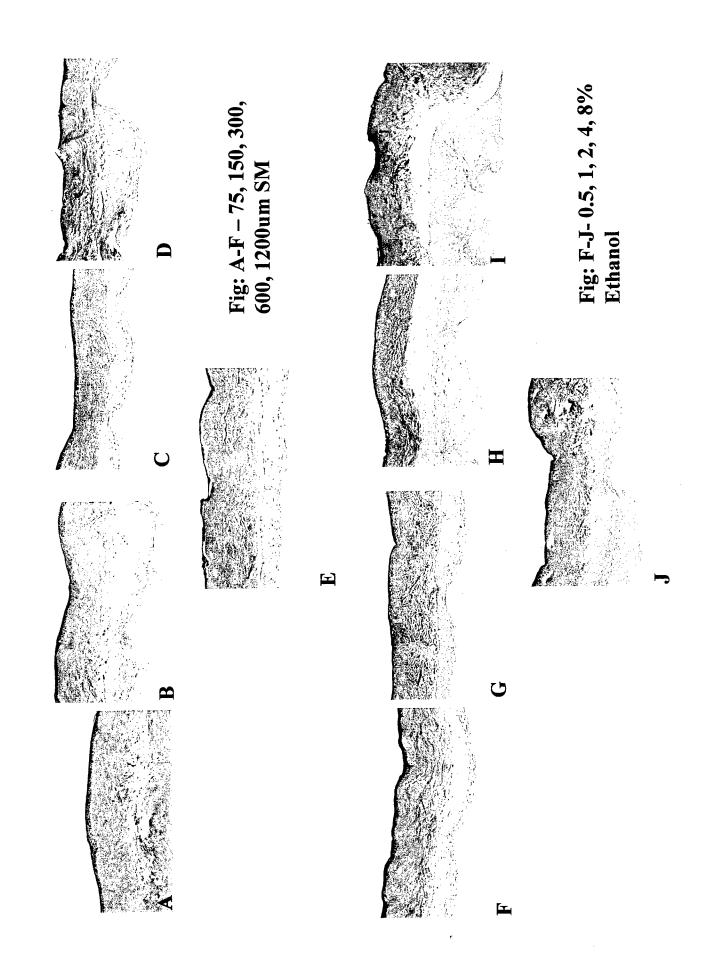


Fig.39: Dose Response to SM shown by NHK's on Alloderm along with controls

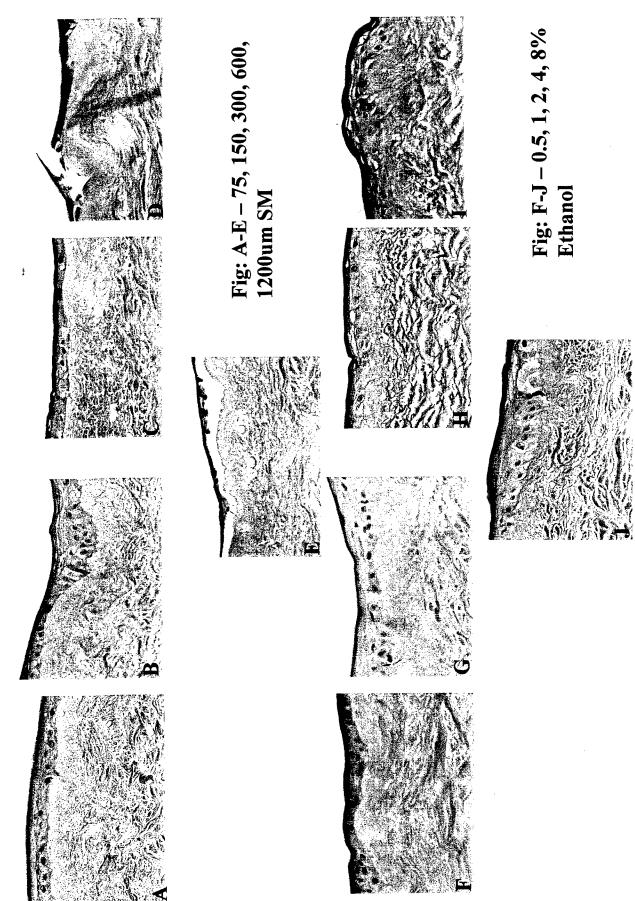
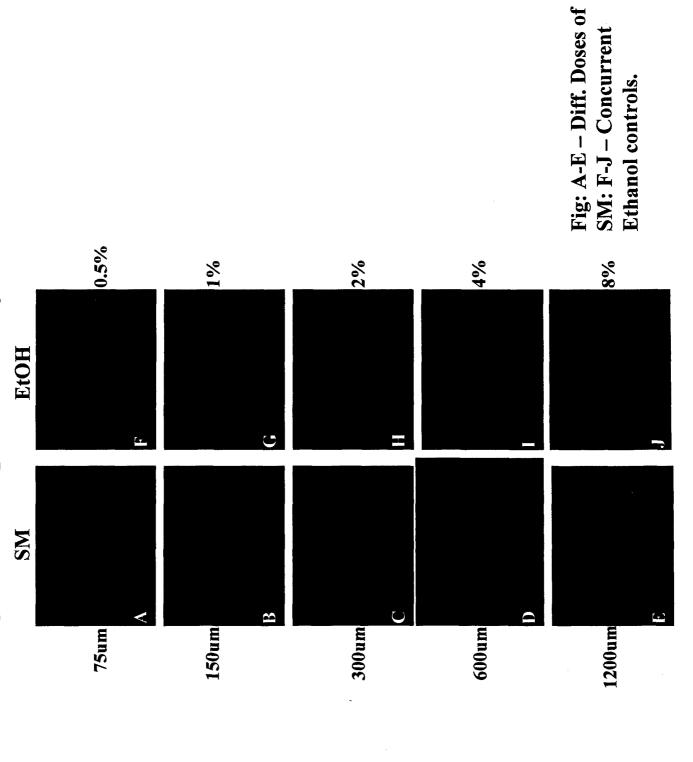


Fig. 40: M30 Staining for dose-response shown by NHKs on Alloderm



## Fig. 41: Apoptotic Counts Alloderm Exposed to diff. Doses of SM along with concurrent controls

## Table:

Apoptotic counts.	19	0	24	S	61	<b>ග</b>	102	17	286	18
Chemical	75u <sub>™</sub> SM	0.5% EOH	150u∾.SM	1% EtOH	300u <sub>2</sub> °SM	2%EtOH	600u <sub>™</sub> SM	4%EtOH	1200um SM	8%EtOH

## Graph:

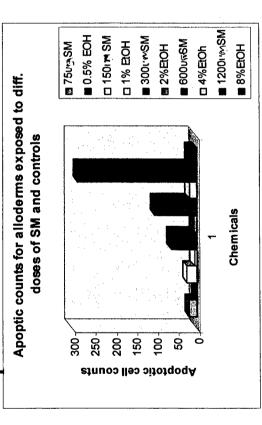


Fig. 42: Effects of SM on diff. Cell types seeded on alloderm along with controls.

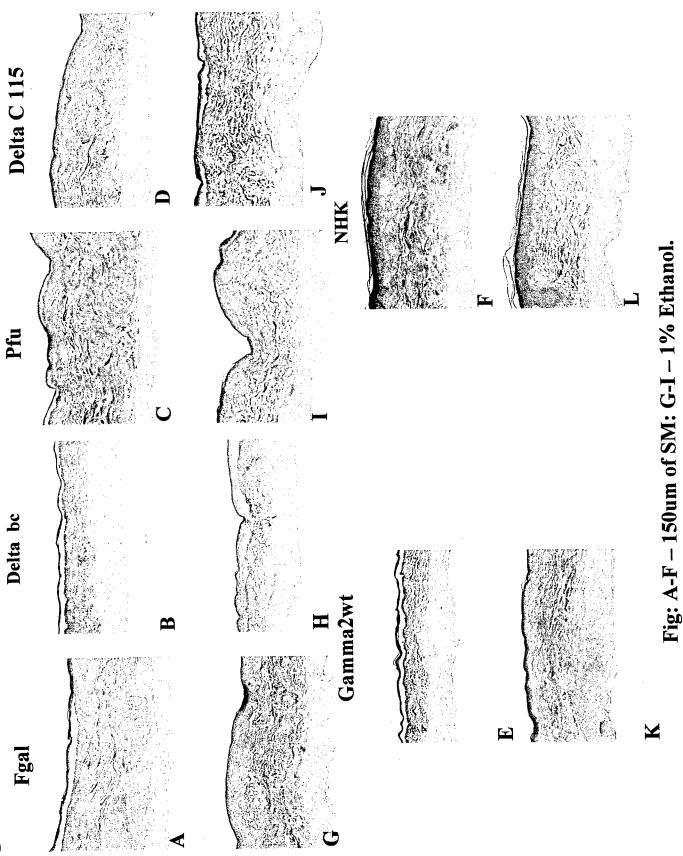


Fig. 43: Effects of SM on diff. Cell types on alloderm along with controls.

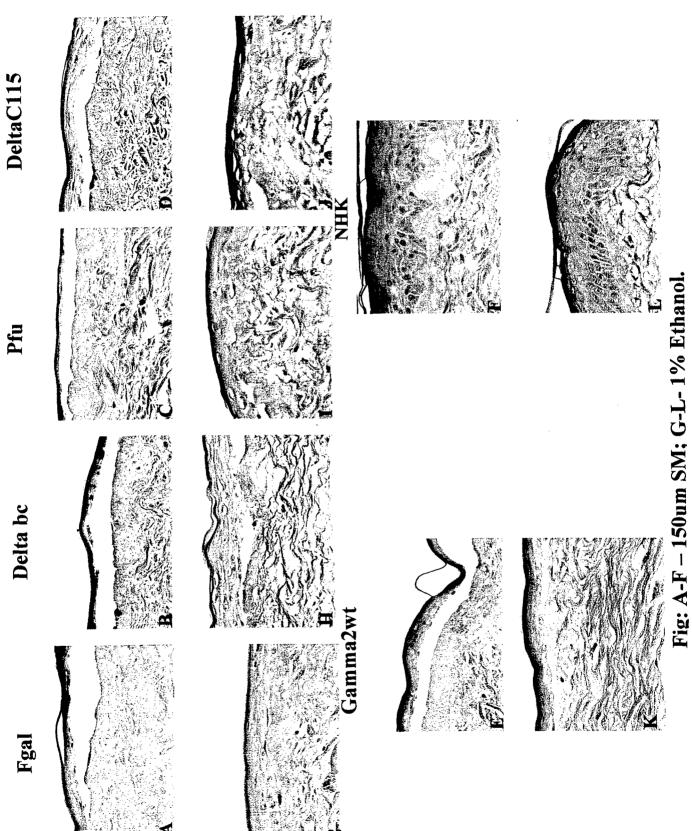


Fig. 44: M30Staining for diff.cell types on alloderm exposed to SM and Ethanol. Delta C115 Pfu **EtOH** SM SM—150um; EtOH-1% Deltabc Gamma2wt Fgal **EtOH** SM

Fig. 45: Apoptosis for diff. Cells on alloderm exposed to SM and Ethanol

Table: Percentage of apoptosis for diff.cell types on alloderm

Gamma2wt NHK	19	S.
	7	4
DeltaC115	17	4
błu	4	က
Deltabc	20	2
fgal		
Chemicals	SM(150uh)	1%EtOH

